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Biohydrogenation of docosahexaenoic acid in the rumen: identification of intermediates and microbes involved

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List of abbreviations

18:2*n*-6	Linoleic acid
18:3*n*-3	Linolenic acid
22:6*n*-3	Docosahexaenoic acid
ARA	Arachidonic acid
CLA	Conjugated linoleic acid
DGGE	Denaturing gradient gel electrophoresis
DMOX	4,4-Dimethyloxazoline
DMSZ	German Collection of Microorganisms and Cell Cultures
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetracetic acid
FA	Fatty acids
FAME	Fatty acid methyl esters
g	Gravitational acceleration
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
ILVO	Institute of Agricultural and Fisheries Research
LCFA	Long chain fatty acids
MUFA	Monounsaturated fatty acids
NGS	Next generation sequencing
OD	Optical density
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acids
rRNA	Ribosomal ribonucleic acid
SFA	Saturated fatty acids
TAE buffer	Tris-acetate-EDTA buffer
UPGMA	Unweighted pair group method with arithmetic mean
uRF	Uncentrifuged-autoclaved rumen fluid
VA	Vaccenic acid
VFA	Volatile fatty acids

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Introduction

Ruminant derived products such as milk and meat are important sources of fat in the human diet. These foods are derived predominantly from farmed ruminants such as cattle, sheep and goats. It is well known that fat of ruminant products is high in saturated fatty acids (SFA) and low in unsaturated fatty acids. In Europe, dairy products and beef provide about 27-57% and 14-29% of the total SFA intake respectively (Shingfield *et al*., 2013). The high intake of certain SFA is possibly one factor in the development of chronic diseases such as metabolic syndrome and type-II diabetes (Funaki, 2009; Riccardi *et al*., 2004). On the other hand, increased intake of unsaturated fatty acids has been shown to provide health benefits to the consumers (Ruxton *et al*., 2004). Clinical studies in human subjects have established that consumption of PUFA such as EPA (20:5*n*-3) and DHA (22:6*n*-3) is associated with improvement in reproductive, retinal, cardiovascular and mental functions in the human population (Baker *et al*., 2016; Cardoso *et al*., 2016; Swanson *et al*., 2012). Additionally, 22:6*n*-3 has been associated with physiological benefits also in dairy cows (Mattos *et al*., 2004). As ruminant-derived foods are a major source of fat in the human diet, several studies have examined the used of 22:6*n*-3 supplementation to growing and lactating cattle to enhance the concentrations of 22:6*n*-3 in meat and milk to improve human health. Altering the fatty acid composition of milk and meat represent one means to lower SFA intake and increase PUFA in the human diet. A feeding strategy to decrease SFA content and increase PUFA content in ruminant products is the supplementation of PUFA sources to ruminants, yet PUFA in milk and meat are little increased due to biohydrogenation in the rumen (Chilliard *et al*.,

2007). The biohydrogenation process has been known for a long time to occur as a result of microbial activity. If the bacteria responsible for the biohydrogenation of PUFA can be controlled, it may be possible to improve the healthiness of ruminant meat and milk by increasing their PUFA content. The biohydrogenation pathways of the main PUFA occurring in the ruminant diet (i.e. 18:2*n*-6 and 18:3*n*-3) have been extensively studied and several bacteria involved in the predominant pathways of 18:2*n*-6 and 18:3*n*-3 biohydrogenation have been identified. The extensive knowledge on the biohydrogenation of 18:2*n*-6 and 18:3*n*-3 is in contrast with the limited knowledge on 22:6*n*-3 biohydrogenation. So far, the bacterial species involved in biohydrogenation of 22:6*n*-3 remain unknown. Characterization of the bacteria involved in 22:6*n*-3 biohydrogenation will contribute to a better understanding of the mechanisms involved in 22:6*n*-3 biohydrogenation in the rumen. A better understanding could lead to opportunities to improve the fatty acid composition of ruminant products, and thereby improve human health.

In the following sections, the definition of lipids and fatty acids, the fate of feed lipids in the rumen, fatty acid saturation as well as the bacteria involved in the known biohydrogenation pathways will be described.

Lipids

Lipids are highly diverse molecules found in both animals and plants. Lipids include a broad range of different lipid groups, and each group has its own functionality. The most important biological functions are i) storage and transport of energy, ii) structural components in cell membranes, and iii) precursors of hormones (Teo *et al*., 2015). In plants, lipids can be classified into two classes: non-polar lipids mainly serving as storage in seeds, and polar lipids associated with cellular membranes. The polar lipids in plants are mainly glycolipids, phospholipids, and sterols, whereas the non-polar lipids are mainly triglycerides (TAG) (Buccioni *et al*., 2012). The basic building block of lipids are fatty acids (FA) and their structure plays a key role in determining the biological function of lipids. Plant lipids are a major source for FA in the ruminant diet and of great interest, as they are precursors for the FA found in ruminant derived foods.

Fatty acids

Fatty acids (FA) consist of the elements carbon (C) hydrogen (H) and oxygen (O) arranged as a carbon chain with a carboxyl group at one end and a methyl group on the other. Classification of FA is based according to their chain length and the number of double bonds. SFA do not contain any double bond and are therefore 'saturated' with hydrogen. When double bonds are present, fatty acids are said to be unsaturated. Unsaturated fatty acids containing two or more double bonds are called polyunsaturated fatty acids (PUFA) (Figure 1).

In the shorthand nomenclature of FA, for example stearic acid, the number of carbon atoms and double bonds are abbreviated "18:0" indicating that the fatty acid acyl

chain is made of 18 carbon atoms without a double bond whereas linoleic acid (18:2) has two double bond. The number before and after the colon indicate the number of carbon atoms and double bonds in the fatty acyl chain, respectively. In addition, there are two ways to explicit the position of the double bonds 1/ counting from the carboxyl terminus the position of the double bonds can be shown by the Greek letter delta (Δ) e.g. Δ9,12-18:2. 2) and 2/ when counting from the methyl end (*n*) the location of the fist double bond can be writer as *n*-x (e.g. 18:2*n*-6). Docosahexaenoic acid named in the shorthand nomenclature 22:6*n*-3 is a PUFA containing 22 carbon atoms and 6 double bonds (Δ4,7,10,13,16,19-22:6) (Figure 1).

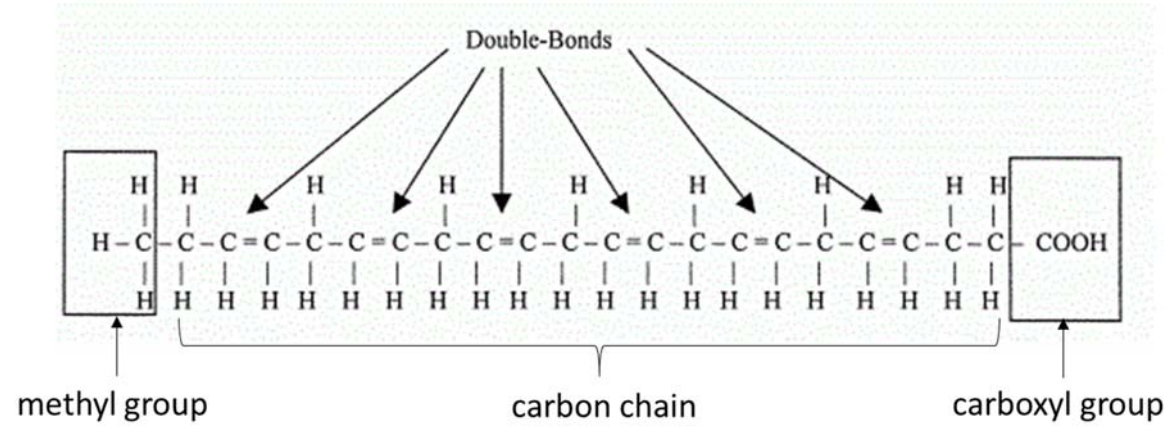

Figure 1. Structure of docosahexaenoic acid (22:6*n*-3; DHA). 22:6*n*-3 contains 22 carbon atoms and 6 double bonds (C=C). At one end is the carboxyl group (–COOH) and at the other end is a methyl group (–CH3).

The double bonds in FA can occur in a *cis* or *trans* configuration. In a *cis* configuration, the two hydrogen atoms adjacent to the double bond are on the same side of the chain and in a *trans* configuration, the two hydrogens are bound to opposite sides of the double bond. In addition, double bonds in unsaturated fatty

acids are generally in the *cis* configuration and separated by a single methylene group (methylene-interrupted saturation), i.e. there is a saturated carbon between two adjacent double bonds. In a systematic way, the configuration and position of the double bonds in the fatty acid chain can be indicated by the term *trans*- or *cis*- followed by the carbon-x were the double bond occurs; starting counting from the carboxyl end. Although in naturally occurring unsaturated fatty acids, double bonds are generally in *cis* configuration, ruminant products have fatty acids of *trans*-geometry formed by ruminal bacteria.

Effects of FA in human health

Fatty acids have broad metabolic functions and exist in free forms or integrated into more complex lipids. Because of their highly reduced chemical structure, fatty acids yield more than twice as much energy upon oxidation compared with saccharides making fat the most efficient form for living organisms to store excess energy. Apart from being an energy source, they have biological activities that act to influence cell and tissue metabolism, function, and responsiveness to hormonal and other signals. Through these effects, fatty acids influence health, well-being, and disease risk. These effects are dependent of the structure of the fatty acids (Ruxton *et al*., 2004).

High intake of saturated FA (SFA), particularly 12:0 (lauric acid), 14:0 (myristic acid) and 16:0 (palmitic acid) increase plasma LDL-cholesterol levels, increase blood coagulation and promote inflammation (Esrey *et al*., 1996; Siri-Tarino *et al*., 2010). SFA are also related to reduced insulin sensitivity, which is a key factor for the development of metabolic syndrome (Hulbert *et al*., 2005). In accordance with these

effects, a high exposure to SFA is associated with higher risk of coronary heart disease, cardiovascular disease and type 2 diabetes (Hulbert *et al*., 2005; Siri-Tarino *et al*., 2010).

The main monounsaturated FA (MUFA) in the human diet are *cis*-9 18:1 (oleic) and *cis*-9 16:1 (palmitoleic acid). When MUFA are supplemented, they have been shown to reduce plasma LDL-cholesterol levels, improve insulin sensitivity and to lower the incidence of type 2 diabetes (Calder, 2015).

The most prevalent *n*-6 polyunsaturated FA (PUFA) in the human diet is 18:2*n*-6 (linoleic acid; LA). It has been demonstrated that 18:2*n*-6 lowers blood cholesterol (Harris *et al*., 2009). suggesting that it may lower cardiovascular disease incidence and mortality (Harris *et al*., 2009; Sakai and Sasaki, 2016). The second most important *n*-6 fatty acid is 20:4*n*-6 (arachidonic acid; ARA) which is synthesized in the body from 18:2*n*-6 by enzymatic desaturation and elongation. 20:4*n*-6 is abundantly present in brain and plays a role in biological functions such as growth and infant development (McNamara *et al*., 2017; St-Onge and Travers, 2016).

An increased intake of *n*-3 PUFA is linked with a reduced risk of cardiovascular disease, cardiac arrhythmias, sudden cardiac death, atherosclerosis, and hypertension. The most important *n*-3 PUFA are 18:3*n*-3 (linolenic acid; LA), 20:5*n*-3 (eicosapentaenoic acid; EPA) and 22:6*n*-3 (docosahexaenoic acid; DHA). 18:3*n*-3 is essential in the diet because it cannot be synthesized in the human body. 18:3*n*-3 can be converted into 20:5*n*-3 and into 22:6*n*-3 by increasing the chain length and increasing the unsaturation by the action of enzymes in humans. However, the

conversion into 20:5*n*-3 and 22:6*n*-3 is limited (Calder, 2015). Therefore, an adequate consumption of 22:6*n*-3 and 20:5*n*-3 is essential. Furthermore, 20:5*n*-3 and 22:6*n*-3 exert a range of biological activities that may reduce tumor growth (Chapkin *et al*., 2008). Cell membranes contain modest amount of 20:5*n*-3 and greater amounts of 22:6*n*-3. Membranes of the brain and eye contain high amounts of 22:6*n*-3 (Calder, 2015). The high content of 22:6*n*-3 in neuron membranes suggest that 22:6*n*-3 plays a role in brain health and cognitive functions. The aging brain declines in cognitive function and may lead to neurodegenerative diseases such as Alzheimer and Parkinson's diseases (Cardoso *et al*., 2016). Dietary supplementation of 22:6*n*-3 has been proved important in ensuring healthy ageing, by enhancing memory and strengthening neuroprotection in general (Cardoso *et al*., 2016; Swanson *et al*., 2012).

Conjugated linoleic acids (CLA) are positional isomers of 18:2*n*-6. They have shown beneficial health effects such as anticarcinogenic, reduction in body fat deposition, reduced development of atherosclerosis, stimulation of immune function, blood glucose lowering and improving bone mass (Fuke and Nornberg, 2017; Gorissen *et al*., 2015). CLA isomers mainly originate from bacterial biohydrogenation of 18:2*n*-6 in the rumen and therefore found naturally in foods from ruminant animals such as meat, milk, and dairy products (Gorissen *et al*., 2015).

Within the category of unsaturated fatty acids there is one subtype that has the opposite effect on cardiovascular disease risk; fatty acids containing one or more double bonds in the *trans* configuration. Similar to SFA, *trans* fatty acids increase

plasma cholesterol concentrations which is associated with higher risk of cardiovascular disease (Lichtenstein, 2014). Moreover, *trans* FA were consistently associated with an increase of inflammation metabolites in blood plasma (Lemaitre *et al*., 2006). Isomeric *trans* forms are produced by bacteria in the rumen and also by the industrial processing of unsaturated FA to develop commercial products. Reports have suggested that dietary *trans* fatty acids produced industrially are more closely associated with cardiovascular disease risk then dietary *trans* fatty acids derived from ruminant fat (Hobbs *et al*., 2016). For instance, dairy fat provides *trans*-11 18:1 (vaccenic acid) and studies have suggested possible beneficial effects on coronary heart disease and cancer (Gebauer *et al*., 2007; Hobbs *et al*., 2016).

Overall, nutritional research has focused on the health promoting benefits on unsaturated FA due to their health promoting effects in humans. Synthesis of LCFA such as 20:4*n*-6, 20:5*n*-3 and 22:6*n*-3 occurs endogenously in the human body, but conversion rates are low and are considered inadequate to provide their health-protective or -promoting benefits. Hence research also focused on the direct supply from different food sources to increase the human intake of PUFA. As ruminant derived-products such as milk and meat are important sources of SFA and poor in PUFA, altering the fatty acid composition of milk and meat represent one means to lower the human intake of SFA and increase the intake of PUFA.

Lipid metabolism in the rumen

Upon entering the rumen, lipids are altered resulting in a marked difference between the fatty acid profile of lipids in the diet (mostly PUFA) and lipids leaving the rumen (mostly SFA) due to bacterial lipolysis and biohydrogenation taking place in the rumen (Harfoot and Hazlewood, 1997). The amount and composition of fatty acids entering the rumen differ among different feedstuff available for ruminants. Therefore, the input of type of FA can be controlled by manipulating the ruminant diet. In grass the major PUFA is linolenic acid (18:3*n*-3) constituting up to 50-75% of the total fatty acids of grasses (Table 1) (Harfoot and Hazelwood 1997). In maize and whole crop silages linoleic acid (18:2*n*-6) is the predominant fatty acid (Table 1). Additional lipids may be supplemented in the form of oils. Linseed oil contains relatively high proportions of 18:3*n*-3, whereas rape and sunflower oils are abundant in 18:2*n*-6. Ruminant diets may also be supplemented with fish oil or marine algae containing 20:5*n*-3 and 22:6*n*-3 (Table 1).

Lipolysis

When dietary lipids enter the rumen, the initial step in lipid metabolism is the hydrolysis of the ester linkages of lipids resulting in non-esterified FA with free carboxyl groups (free FA) in the rumen (Jenkins *et al*., 2008). The extent of hydrolysis is between 85% and 95% (Doreau and Ferlay, 1994).

Table 1. Fatty acid composition of forages, oilseeds, plant and marine lipid supplements commonly used in ruminant diets.

Source		Fatty acids composition (g/100g fatty acids)						
		16:0	18:0	*cis*-9 18:1	18:2*n*-6	18:3*n*-3	20:5*n*-3	22:6*n*-3
Grass	Fresh	15.1	2.3	4.4	18.2	49.9	–	–
	Silage	20.1	2.1	2.5	14.2	50.4	–	–
	Hay	35.0	0.6	2.6	5.4	15.6	–	–
Red clover	Fresh	21.5	7.1	4.2	17.3	35.6	–	–
Lucerne	Hay	22.9	4.1	4.3	17.7	22.3	–	–
Maize	Silage	17.4	2.2	20.3	44.8	6.6	–	–
Rape	Oil	6.0	2.3	48.1	27.4	10.3	–	–
	Whole seeds	4.8	2.0	56.8	19.3	8.3	–	–
Sunflower	Oil	6.1	3.6	26.5	60.4	0.1	–	–
	Whole seeds	5.1	4.3	21.6	66.8	0.2	–	–
Linseed	Oil	4.2	2.7	16.5	15.8	57.8	–	–
	Whole seeds	6.1	3.4	18.8	16.3	54.4	–	–
Fish oil		15.0	2.6	11.0	1.2	0.9	16.5	10.5
Marine Algae		26.3	0.9	1.1	0.3	0.2	0.1	37.8

Adapted from Clapham et al. (2005) and Shingfield and Wallace (2014)

Microbial lipases are mainly responsible for the lipolytic activity in the rumen (Dawson and Hemington, 1974; Dawson *et al*., 1977). The most active lipolytic species isolated from the rumen is *Anaerovibrio lipolyticus* (Hobson and Mann, 1961). Its relative 16S rRNA gene abundance in the rumen is around 0.05% (Minuti *et al.* 2015). This bacterium hydrolyses triglycerides found in cereal grains and plant oils but lacks the ability to hydrolyse galacto- and phospholipids found in forages (Prins *et al*., 1975). Forage lipids are hydrolysed by *Butyrivibrio fibrisolvens* spp. (Hazlewood and Dawson, 1975) to yield diacyglycerides that are further degraded to monoacylglycerides and subsequently to free FA and glycerol (Dawson and Hemington, 1974). Lipolysis is a crucial step for biohydrogenation of unsaturated FA, as the presence of a free carboxyl group is a prerequisite for biohydrogenation to take place (Hazlewood *et al*., 1976) (Harfoot and Hazlewood, 1997).

More recently, lipolytic activity was reported for other rumen bacteria belonging to the *Clostridium, Propionibacterium, Staphylococcus* and *Selenomonas* genera (Edwards *et al.* 2013).

The lipase from *A. lipolyticus* was first studied by Henderson (1971) and more recently by Privé *et al.* (2013). Its genome contains three genes coding for lipases (Privé *et al.,* 2013). Recent metagenomic study revealed 14 novel lipases from a bovine rumen metagenome. (Privé *et al.*, 2015).

Biohydrogenation

Biohydrogenation of unsaturated FA is the second major transformation of dietary lipids in the rumen. During this process, PUFA are gradually converted into SFA. Evidence of ruminal biohydrogenation was first reported by Reiser (1951). When linseed oil was incubated with sheep ruminal contents. The amount of 18:3*n*-3 decreased from 30% to 5% with a concomitant increase in 18:2 (Reiser, 1951).

Bacteria responsible of biohydrogenation of 18-carbon PUFA

Initially, the bacterium *B. fibrisolvens* was the only rumen bacterium known capable of carrying out biohydrogenation of 18-carbon PUFA (i.e. 18:2*n*-6 and 18:3*n*-3). This bacteria, is able to convert 18:2*n*-6 mainly into *cis*-9, *trans*-11 18:2 intermediate and subsequently to reduce the *cis*-9, *trans*-11 18:2 to *trans-11*-18:1 (Polan *et al*., 1964). Later, *Butyrivibrio hungatei* was also found to convert 18:2*n*-6 to *trans-11*-18:1 (Maia *et al*. 2007). *Clostridium proteoclasticus* is the only species known to produce 18:0 (Figure 2) (Wallace *et al*., 2006). *C. proteoclasticus* was reclassified as *Butyrivibrio proteoclasticus* based on its phylogenetic, morphologic and physiologic characteristics (Moon *et al*., 2008). Classification of biohydrogenating bacteria was proposed by Kemp and Lander (1984). The classification consisted into two different groups, group A and group B, based on their biohydrogenation products (Kemp and Lander, 1984). The group B bacteria carry out the complete reduction of 18:2*n*-6 to 18:0. Group A bacteria are responsible for the isomerization to the conjugated diene and hydrogenation to 18:1 isomers and are unable to complete the biohydrogenation of the *trans*-11-18:1 intermediate to 18:0. An example of a group A bacteria is *B.*

fibrisolvens whereas group B is represented by B. *proteoclasticus* (Figure 2). (Harfoot and Hazlewood, 1997).

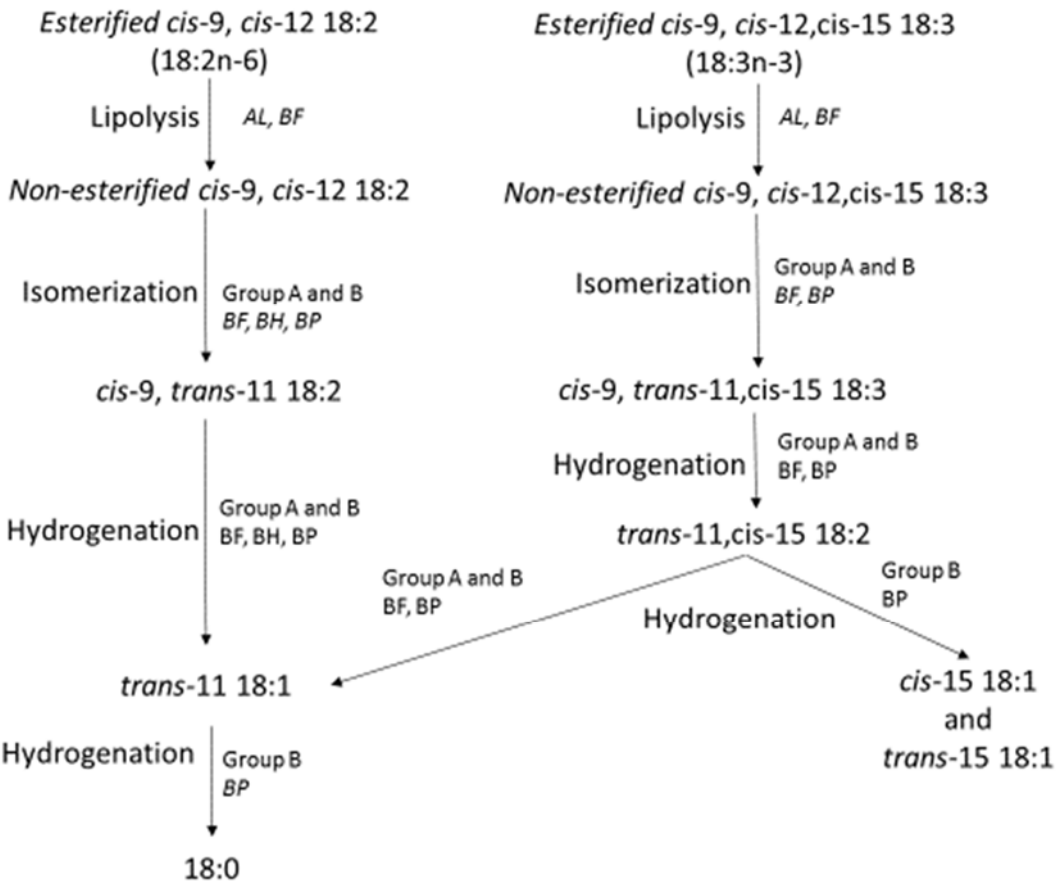

Figure 2. Scheme for the main biohydrogenation pathway of 18:2*n*-6 and 18:3*n*-3 and the rumen bacteria involved in each biohydrogenation reaction. AL: *Anaerovibrio lipolytica*, BF: *Butyrivibrio fibrisolvens*, BH: *Butyrivibrio hungatei*, BP: *Butyrivibrio proteoclasticus.* Figure adapted from Harfoot and Hazewood (1997).

Other rumen bacterial species identified with hydrogenating capacity are *Megasphaera elsdenii* and *Propionibacterium acne*s which produce *trans*-10, *cis*-12 18:2. These FA are formed via alternative pathways of biohydrogenation of 18-

carbon PUFA (Table 2) (Kim *et al*., 2002; Wallace *et al*., 2007). Under normal situation, these FA are present at much lower concentrations than the main intermediates. The capacity of *Megasphaera elsdenii* to hydrogenate 18:2*n*-6 was not confirmed by Maia *et al*., (2007).

Besides rumen bacteria, microorganisms isolated from other environments (e.g. mice and human colon, waste water) have shown to metabolize PUFA resulting in diverse fatty acids. These bacteria belong to genera Lactobacilli, Propionibacteria, Bifidobacteria and Clostridia (Gorissen *et al*., 2015; Sakurama *et al*., 2014).

Table 2. Rumen bacterial species and intermediates produced by alternative or minor biohydrogenation pathways of 18-carbon PUFA

Bacterium	Substrate	Product formed
E. faecalis	*cis*-9 18:1	10-OH 18:0
	cis-9, *cis*-12 18:2	10-OH *cis*-12 18:1, 13-OH *cis*-9 18:1
P. acnes	*cis*-9 18:1	10-OH 18:0, 10-O 18:0
	trans-10 18:1	10-OH 18:0, 10-O 18:0
	cis-9, *cis*-12 18:2	*trans-10, cis-12* 18:2, *cis-10, trans*-12 18:2*, trans-10, trans*-12 18:2
M. elsdenii	*cis*-9, *cis*-12 18:2	*trans-10, cis-12* 18:2
S. ruminantium	*cis*-9 18:1	10-OH 18:0
C. aminophilum	*cis*-9, *cis*-12 18:2	*cis*-9 18:1
M. multiacidus	*cis*-9, *cis*-12 18:2	*cis*-9 18:1
S. bovis	*cis*-9, *cis*-12 18:2	13-OH *cis*-9 18:1

Adapted from Shingfield et al. 2014.

Biohydrogenation of 18-carbon PUFA

Rumen biohydrogenation of 18:2*n*-6 and 18:3*n*-3 is extensive. It ranges from 70 to 95% for 18:2*n*-6 and from 85 to 100% for 18:3*n*-3 with 18:0 being the major fatty acid leaving the rumen for most diets (Harfoot and Hazlewood 1997; Chilliard *et al.* 2007). Characterization of biohydrogenation pathways *in vivo* represents a major challenge due to PUFA containing feed are composed by diverse types of PUFA rather than one single PUFA, making it difficult to draw conclusion on the origin of a specific biohydrogenation intermediate. In this regard, incubations of fatty acids with rumen fluid or pure cultures of rumen bacteria have proven crucial in establishing the main and putative intermediates formed during the biohydrogenation of 18:2*n*-6 and 18:3*n*-3. Early studies showed that incubation of *B. fibrisolvens* with 18:2*n*-6 was first converted to a conjugated 18:2 FA isomer (putatively *cis*-9, *trans*-11 18:2) giving the first indication of the initial biohydrogenation reaction. This conjugated isomer was then hydrogenated to *trans*-11 18:1 with no further metabolism. (Kepler *et al*., 1966; Polan *et al*., 1964). Kepler and Tove (1967) confirmed that the main pathway of rumen biohydrogenation of 18:2*n*-6 (*cis*-9, *cis*-12 18:2) starts with an isomerization of the *cis*-12 bond to yield the conjugated fatty acid *cis*-9, *trans*-11-18:2. The second step consist in the actual reduction resulting in *trans*-11 18:1 (vaccenic acid; VA) and then in 18:0 (stearic acid) (Kepler and Tove 1967) (Figure 2).

Similarly, biohydrogenation of 18:3*n*-3 (*cis*-9, *cis*-12, *cis*-15 18:3) yields the conjugated triene *cis*-9, *trans*-11, *cis*-15 18:3, which is then sequentially hydrogenated into *trans*-11,*cis*-15 18:2, *trans*-11 18:1 and finally into 18:0 when incubated with mixed cultures (Harfoot and Hazlewood 1997). Initial studies also administered 18:2*n*-6 labelled using ^{14}C isotopes to track the fate of 18:2*n*-6 (Ward *et*

al., 1964; Wood *et al*., 1963). Wood et al. (1963) observed that upon ^{14}C-18:2*n*-6 supplementation in the rumen up to 55% was recovered in 18:1 FA and 46% was fully saturated to 18:0 (Wood *et al*., 1963). Subsequent studies showed that next to the main biohydrogenation pathway of 18-carbon PUFA, biohydrogenation yields a wide variety of other biohydrogenation intermediates, in particular *cis* and *trans* isomers of 18:2 and 18:1 (Chilliard *et al*., 2007; Duckett *et al*., 2002; Loor *et al*., 2002) (Figure 2 and 3).

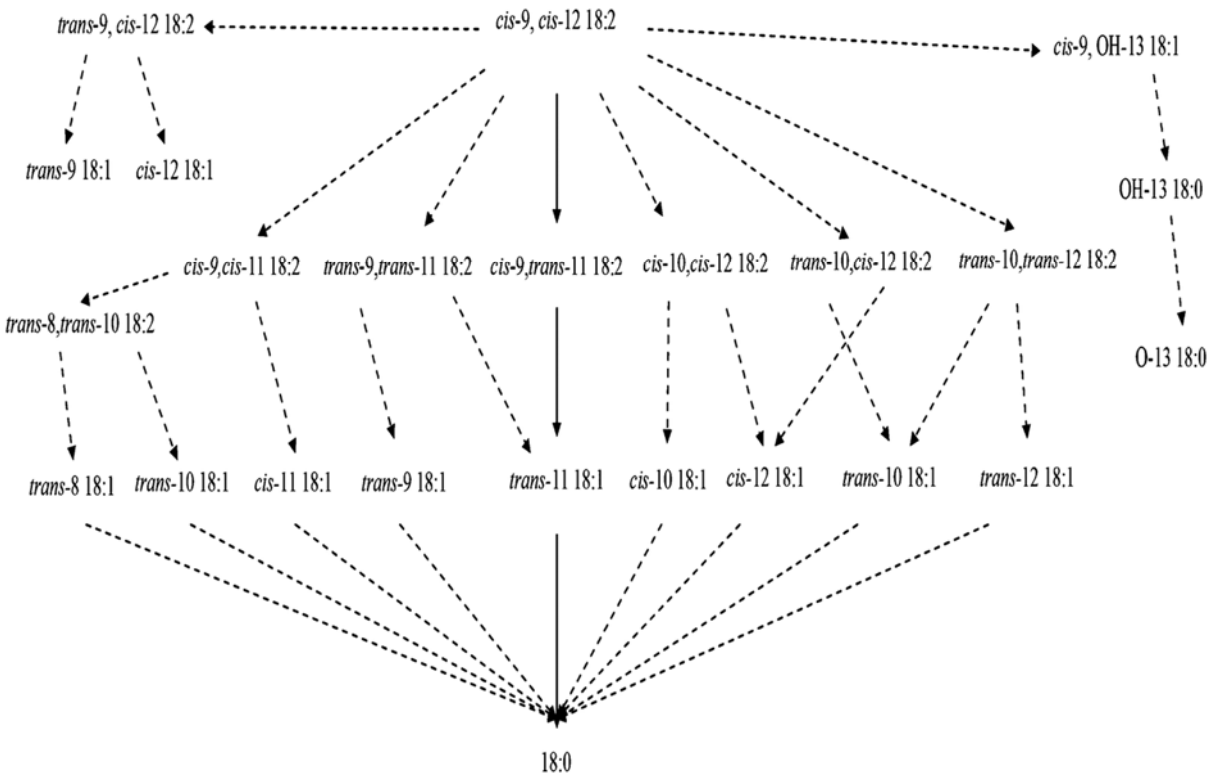

Figure 2. Main and secondary pathways of *cis*-9, *cis*-12 18:2 metabolism in the rumen. Solid lines represent the major biohydrogenation pathway; dashed lines the formation of minor fatty acid metabolites (Shingfield and Wallace, 2014).

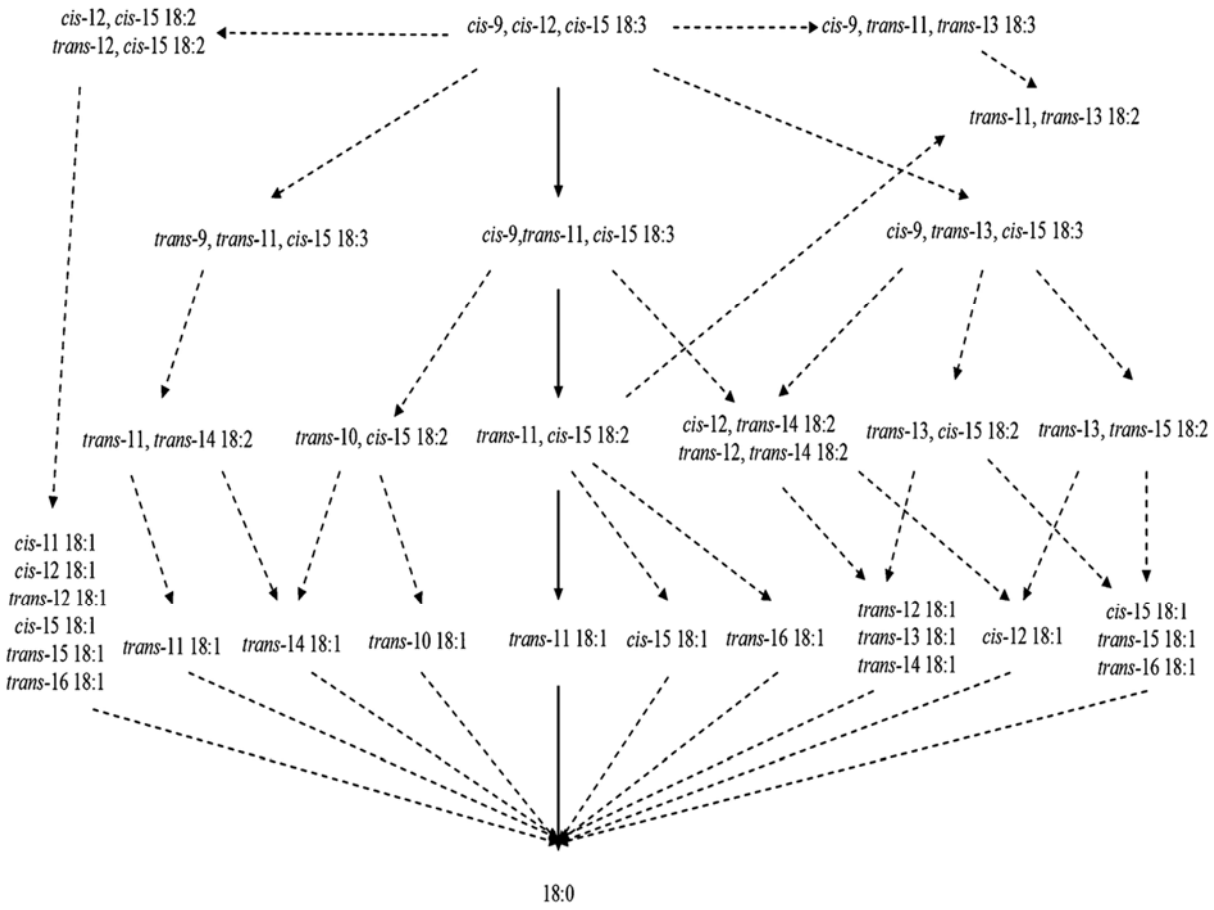

Figure 3. Main and secondary pathways of *cis*-9, *cis*-12, *cis*-15 18:3 metabolism in the rumen. Solid lines represent the major biohydrogenation pathway; dashed lines the formation of minor fatty acid metabolites (Shingfield and Wallace, 2014).

Further characterization of these pathways emerged from *in vitro* incubations of mixed rumen cultures with pure 18:2*n*-6, 18:3*n*-3 and linseed oil with quantification of the fatty acids over a 24-h incubation (Jouany *et al*., 2007). The large number of intermediates identified in such studies (Chilliard *et al*., 2007; Honkanen *et al*., 2012; Jouany *et al*., 2007; Wallace *et al*., 2007) suggests that the biohydrogenation pathways of 18:2*n*-6 and 18:3*n*-3 are much more complex than originally thought (Figure 2 and 3).

Isomerization

The first committed step of 18:2*n*-6 and 18:3*n*-3 biohydrogenation in the rumen is an isomerization reaction. This isomerization is enzymatically catalysed by linoleate isomerase which was found to be present in the membrane of *Butyrivibrio* spp (Kepler and Tove, 1967). The main conjugated isomers formed from metabolism of 18:3*n*-3 and 18:2*n*-6 are *cis*-9, *trans*-11, *cis*-15 18:3 and *cis*-9, *trans*-11 18:2 respectively. Flows of *cis*-9, *trans*-11 18:2 in the duodenum of sheep have been reported to vary between 0.12 and 0.20 g/day (Kucuk *et al*., 2001) depending on the diet. Ruminal outflow of *cis*-9, *trans*-11 18:2 in non-lactating cows (0.3–0.5 g/day; Lock and Garnsworthy 2002), and lactating cows (0.2–1.7 g/day; Piperova *et al*., 2002; Shingfield *et al*., 2003) have also been reported.

In addition to formation of *cis*-9, *trans*-11 18:2 an important secondary pathway of 18:2*n*-6 biohydrogenation in the rumen was found when dairy cows were fed high concentrate diets (Griinari *et al*., 1998). In this pathway, 18:2*n*-6 is metabolized to *trans*-10, *cis*-12 18:2 instead of *cis*-9, *trans*-11 18:2 and *trans*-10, *cis*-12 18:2 is further converted to *trans*-10-18:1 in the rumen. It was proposed that this secondary pathway of 18:2*n*-6 metabolism was carried out by bacteria that were tolerant lo tow ruminal pH which occurs due to the rapid fermentation of the concentrate diet (Griinari *et al*., 1998). Later, pure culture studies demonstrated production of *trans*-10, *cis*-12-18:2 by two acid tolerant bacteria, *Megasphaera elsdenii* and *Propionibacterium acnes* (Table 2) (Kim *et al*., 2002; Wallace *et al*., 2007). Numerous other conjugated 18:2-isomers have been documented in *vivo* and *in vitro*. These isomers are present at much lower concentrations, and they differ by position (e.g., 7–9, 8–10, 9–11, 10–12, 11–13, or 12-14) or geometric orientation

(*cis-trans, trans-cis, cis-cis, and trans-trans*) of the double bond pair (Table 3) (Palmquist *et al*., 2005).

Table 3. Distribution and ruminal outflow of *trans* 18:1 and isomers of conjugated 18:2 fatty acids in growing and lactating cattle

Conjugated 18:2		*trans* 18:1	
Isomer	Ruminal outflow (g/day)	Isomer	Ruminal outflow (g/day)
trans-7 ,*cis*-9	<0.01	*trans*-4	0.5-0.7
trans-7,*trans*-9	<0.01-0.05	*trans*-5	0.4-0.6
trans-8, *cis*-10	0.01-0.02	*trans*-6-8	0.4-6.7
trans-8, *trans*-10	<0.01-0.10	*trans*-9	0.8-6.2
cis-9, *cis*-11	<0.01-0.01	*trans*-10	1.7-29.1
cis-9, *trans*-11	0.19-2.86	*trans*-11	5.0-121.0
trans-9, *trans*-11	0.22-0.55	*trans*-12	0.5-9.5
trans-10, *cis*-12	0.02-0.32	*trans*-13-14	6.5-22.9
trans-10, *trans*-12	0.05-0.06	*trans*-15	3.2-8.5
cis-11, *trans*-13	0.01-0.10	*trans*-16	3.1-8.0
trans-11, *cis*-13	0.01-0.46		
trans-11, *trans*-13	0.09-0.40		
cis-12, *trans*-14	<0.01-0.05		
trans-12, *trans*-14	0.08-0.19		

Adapted from Palmquist et al. (2005)

Pure culture studies showed *B. fibrisolvens* to produce *trans*-9, *trans*-11-18:2 but the amount produced was rather small (Wallace *et al*., 2007). Ruminal synthesis of *trans*-9, *trans*-11-18:2 and *trans*-10, *cis*-12-18:2 are increased with high concentrate diets or when fish oil is present in the diet (Piperova *et al*. 2002; Shingfield *et al*. 2003). Several other studies with pure cultures were carried to establish the role of

bacterial species in the formation of specific conjugated fatty acid isomers of 18:2*n*-6 and 18:3*n*-3 isomerization. These bacteria include *E. faecalis, P. acnes, M. elsdenii, S. ruminantium, C. aminophilum, M. multiacidus* and *S. bovis* (Table 2).

Hydrogenation

Conjugated 18:2 fatty acid isomers formed during the initial isomerization of 18:2*n*-6 are transient. Following isomerization, these intermediates undergo hydrogenation carried out by reductases produced by the ruminal bacteria. Hydrogenation results in the conversion of an unsaturated double bond to a saturated single bond. In the case of *cis*-9, *trans*-11 18:2, the cis-9 double bond is saturated leading to the formation of *trans*-11-18:1, which is the major accumulating intermediate of 18:2*n*-6 and 18:3*n*-3 biohydrogenation. However, biohydrogenation of conjugated 18:2 isomers yields a wide range of both *trans* (-4, -5, 6 -8, -9, -10, -11, -12, -13 and -14) and *cis* (-9, -10, -11, -12 and -13) 18:1 intermediates (Honkanen *et al*., 2012; McKain *et al*., 2010). The subsequent saturation of the double bond leads to the formation of 18:0 as the end product of 18:2*n*-6 and 18:3*n*-3 biohydrogenation.

Biohydrogenation pathways of 18-carbon PUFA have been shown to be affected by conditions of the rumen environment e.g. high concentrate diets caused a decrease in pH from 6.4 to 5.6 which resulted in *trans*-10-18:1 accumulation at the expense of *trans*-11 18:1 (Fuentes *et al*., 2009). These biohydrogenation pathways are also altered when other PUFA such as 22:6*n*-3 are present. AbuGazaleh and Jenkins (2004b) observed several changes in the fatty acid profile when 18:2*n*-6 was incubated with 22:6*n*-3. Addition of 22:6*n*-3 increased the accumulation of *trans* 18:1 isomers and inhibited the biohydrogenation of 18:2*n*-6 to 18:0 (AbuGhazaleh and

Jenkins, 2004a). In dairy cows, supplementation of fish oil increased the duodenal content of 18:1 fatty acids from 13% to 36% while 18:0 was reduced from 54% to 8% (Doreau and Chilliard, 1997). Another source of 22:6*n*-3 is microalgae which was investigated by Boeckaert et al. (2007). When 22:6*n*-3-edible microalgae was incubated *in vitro* with rumen fluid, it inhibited the biohydrogenation of 18:2*n*-6.

Biohydrogenation of 22:6n-3

Marine products derived from fish or algae are the major sources of docosahexaenoic acid (22:6*n*-3) in ruminant diets. In several studies, fish oil or algae was added to the diet to increase the absorption of 22:6*n*-3. However, 22:6*n*-3 was found to be highly metabolized in the rumen. In their review, Fievez et al. (2007) reported *in vivo* biohydrogenation of 763 ± 134 g/kg with supplementation of fish oil or marine algae. *In vitro* incubations with mixed rumen cultures also showed 22:6*n*-3 disappearance (225 ± 242 g/kg) however to a lesser extent as compared to *in vivo* supplementation of fish oil or algae (Fievez *et al*., 2007). The biohydrogenation of 22:6*n*-3 in incubations with mixed ruminal microorganism has been found to decrease in a dose dependent manner (AbuGhazaleh and Jenkins, 2004b; Aldai *et al*., 2012; Fievez *et al*., 2007). Hence, extensive biohydrogenation has been obtained only when supplementing small amounts of 22:6*n*-3 *in vitro* (Aldai *et al*., 2012; Vlaeminck *et al*., 2014). For instance, in the study by Vlaeminck et al., (2014) about 90% of 22:6*n*-3 disappeared when 22:6*n*-3 was added at 0.05mg/mL to the mixed cultures in contrast with 30% disappearance with 0.3mg/mL of 22:6*n*-3.

The biohydrogenation pathways of 22:6*n*-3 have not been elucidated. Analysis of duodenal content of cows supplemented with fish oil showed the ruminal outflow of several 22-carbon fatty acids which were not present in the diet (Kairenius, Toivonen, and Shingfield 2011; Shingfield *et al*. 2012). By analogy to the 18-carbon PUFA biohydrogenation pathway, it was proposed that the metabolic pathway of 22:6*n*-3 would start with an isomerisation step to yield 22:6 isomers with one double bond of *trans*-geometry. The subsequent step would be a reduction reaction to yield a 22:5 intermediate and ultimately the formation of 22:0. (Kairenius, Toivonen, and Shingfield 2011, Jenkins *et al*. 2008). Nevertheless, formation of 22:0 from 22:6*n*-3 in ruminal batch cultures has been reported, but only in trace amounts (Klein and Jenkins, 2011).

Bacteria responsible of biohydrogenation of 22:6n-3

Bacterial species responsible for the biohydrogenation of 22:6*n*-3 are still unknown. Despite the attempts, isolation of bacteria able to biohydrogenate 22:6*n*-3 failed so far probably because biohydrogenating bacteria have been shown to be the most sensitive to the toxic effects of PUFA present in the culture media. PUFA are known to be toxic to many microorganisms including those in the rumen. The mode of action of PUFA antimicrobial activities is not yet clear, but the prime target seems to be the bacterial cell membrane and the various essential processes that occur within and at the membrane. Some of the detrimental effects is related to amphipathic structure, allowing them to interact with the cell membrane to create transient or permanent pores of variable size (Desbois and Smith, 2010). Free fatty acids may also affect bacterial energy production by disrupting the electron transport chain

and/or interfering with oxidative phosphorylation (Desbois and Smith, 2010). Other processes that may contribute to bacterial growth inhibition or death include cell lysis, inhibition of enzyme activity, impairment of nutrient uptake and the generation of toxic peroxidation and autoxidation products (Desbois and Smith, 2010). The antibacterial activity of FFA is influenced by its structure and shape, which is a function of the length of the carbon chain and the presence, number, position and orientation of double bonds (Desbois and Smith, 2010). In general, the number of double bonds is correlated with the degree of toxicity with cis double bonds having a greater effect compared with trans double bonds. As a result of the toxic effects of PUFA, the bacteria could be affected by complete growth inhibition, decrease in cell growth or its delay. Maia et al. (2007) investigated PUFA metabolism of 26 predominant rumen bacterial species and found none of them able to metabolize 22:6*n*-3 (Maia *et al*., 2007). The presence of PUFA including 18:2*n*-6 and 22:6*n*-3 showed complete growth inhibition of some species, whereas other species were able to grow, however to a much lower extent. In contrast, members of the genera *Prevotella*, *Megasphaera*, *Selenomonas*, *Veillonella* and *Anaerovibrio* were insensitive to the presence of 22:6*n*-3 (50µg/mL) (Table 4). Growth seems an important feature as it was shown that bacterial growth was a prerequisite to perform biohydrogenation of 18:2*n*-6 to 18:0 (Wallace *et al*., 2006). Hence failure of biohydrogenating bacteria to grow in the presence of 22:6*n*-3 probably was the mayor reason of lack of 22:6*n*-3 biohydrogenation in previous experiments.

Table 4. Bacteria studied for their biohydrogenation metabolism and growth response in presence of 18:2*n*-6 and 22:6*n*-3*

Species	Metabolism of 18:2*n*-6 (50µg/mL)	Growth affected by the presence of 18:2*n*-6 (50µg/mL)			Growth affected by the presence of 22:6*n*-3 (50µg/mL)		
		Unaffected	Delayed	Inhibited	Unaffected	Delayed	Inhibited
*B. fibrisolvens*JW11	+	+				+	
B. hungatei JK611	+			+			+
*C. aminophilum*49906	+		+			+	
*C. proteoclasticum*P-18	+		+				+
*F. succinogenes*S85	+	+			+		
L. multipara D15d	+		+			+	
*M. multiacidus*46/5	+	+			+		
*P. anaerobius*27336	+		+		+		
S. bovis ES1	+		+			+	
A. lipolytica 5S		+			+		
M. elsdenii LC1		+			+		
P. bryantii B14		+			+		
P. ruminicola 23		+			+		

Table 4 (*Continued*)

Species	Metabolism of 18:2*n*-6 (50µg/mL)	Growth affected by the presence of 18:2*n*-6 (50µg/mL)			Growth affected by the presence of 22:6*n*-3 (50µg/mL)		
		Unaffected	Delayed	Inhibited	Unaffected	Delayed	Inhibited
*R. amylophilus*WP225		+			+		
*S. ruminantium*Z108		+			+		
V. parvula L59		+			+		
P. brevis GA33			+		+		
*E. pyruvativorans*I-6			+			+	
P. albensis M384			+			+	
P. ruminis A12-1			+			+	
P. xylanovorans Mz5			+			+	
*E. ruminantium*2388				+			+
R. albus SY3				+			+

* metabolism of 22:6*n*-3 was not observed in any culture

Adapted from Maia et al. (2007)

Objective of PhD research

The poly-unsaturated fatty acid 22:6*n*-3 has been associated with physiological benefits in many species, including human and dairy cows. The amount of 22:6*n*-3 available for absorption in the small intestine of the ruminant can be increased by intake of marine products (e.g. fish oil, marine algae). However, extensive biohydrogenation in the rumen leaves little 22:6*n*-3 for absorption. Both the bacterial species involved in biohydrogenation of 22:6*n*-3 and the metabolic pathways of this biohydrogenation remain unknown. Characterization of the fatty acids formed during 22:6*n*-3 biohydrogenation and the bacteria involved in this process is important for a more fundamental understanding of the biohydrogenation of 22:6*n*-3 in the rumen. It should be noted that similar statements can be made regarding 20:5*n*-3: biohydrogenation of 20:5*n*-3 extensively occurs in the rumen but the bacteria involved and metabolic pathways of 20:5*n*-3 biohydrogenation are unknown. Knowledge of the metabolism of both fatty acids is important. As much of the work during this PhD is related to optimization of the culture conditions, only one fatty acid was selected for this. Because former research at our lab focused on 22:6*n*-3 rich algae, the current research focused on 22:6*n*-3 rather then 20:5*n*-3.

The main objectives of the PhD thesis are to identify ruminal bacteria involved in the biohydrogenation of 22:6*n*-3 and to characterize fatty acids formed during this process. For this, two different approaches are followed. In one approach, pure cultures of rumen bacteria are used to test their capacity for 22:6*n*-3 biohydrogenation. In a second approach, rumen fluid containing mixed cultures of

rumen bacteria is serially diluted in order to identify the least complex consortium able to perform 22:6*n*-3 biohydrogenation.

Difficulties in isolating these microorganisms are related to the fact that biohydrogenating bacteria are sensitive to the toxic effects of unsaturated FA present in the *in vitro* cultures (Harfoot and Hazlewood, 1997). Thus, in order to be able to fulfil the objectives of this PhD research, adjustment of the incubation conditions to reduce the toxicity might be one strategy to stimulate biohydrogenation of 22:6*n*-3.
For the next chapters of this dissertation, the outline of the research can be summarized as follows:

Chapter 1 focused on optimization of *in vitro* conditions for microbial metabolism of 22:6*n*-3. The hypothesis was that the addition of adsorbants provides an alternative site for adsorption of 22:6*n*-3, decreasing its adherence to bacterial cells and hence lowering its adverse effects on growth and metabolic activity.
Research question: Does the addition of adsorbent compounds stimulate biohydrogenation of 22:6*n*-3 by mixed cultures of rumen bacteria?

Chapter 2 describes a serially dilution approach to select the simplest biological consortium able to biohydrogenate 22:6*n*-3. For this, adjustments to the culture media by addition of uncentrifuged-autoclaved rumen fluid are studied to allow dilution of the rumen inoculum to a level at which the complexity of the microbial community could be largely reduced. Changes in total bacterial populations are analysed using molecular techniques and key bacterial species identified as potential candidates for biohydrogenation of 22:6*n*-3.

Research questions: Can the addition of autoclaved rumen fluid improve biohydrogenation of 22:6*n*-3 in highly diluted rumen inocula? and Can this dilution approach help in identifying bacteria involved in biohydrogenation of 22:6*n*-3?

Chapter 3 reports the pure culture studies to identify bacterial species able to metabolize 22:6*n*-3 and the intermediate products formed during biohydrogenation of 22:6*n*-3.

Research questions: Are the known biohydrogenating bacteria *B. fibrisolvens* and *B. proteoclasticus* able to hydrogenate 22:6*n*-3, and which products are formed?

Finally, a general discussion and future perspectives are presented.

Chapter 1: effect of adsorbants on in vitro biohydrogenation of 22:6*n*-3 by mixed cultures of rumen microorganisms

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ABSTRACT

Studies on microbial biohydrogenation of fatty acids in the rumen are of importance as this process lowers the availability of nutritionally beneficial unsaturated fatty acids for incorporation into meat and milk but also might result in the accumulation of biologically active intermediates. The impact was studied of adsorption of docosahexaenoic acid (22:6*n*-3) to particulate material on its disappearance during 24-h *in vitro* batch incubations with rumen inoculum. Four adsorbants were used in two doses (1 and 5 mg/mL of mucin, gum arabic, bentonite or silicic acid). Additionally, the distribution of 22:6*n*-3 in the pellet and supernatant of diluted rumen fluid was measured. Both mucin and gum arabic increased the recovery of 22:6*n*-3 in the supernatant, indicating that these compounds lowered the adsorption of the fatty acid to ruminal particles. This was associated with an increased disappearance of 22:6*n*-3, when initial 22:6*n*-3 was 0.06 or 0.10 mg/mL, and an increased formation of 22:0, when initial 22:6*n*-3 was 0.02 mg/mL, during the 24-h batch culture experiment. Addition of gum arabic to pure cultures of *B. fibrisolvens* or *B. proteoclasticus* did not negate the inhibitory effect of 22:6*n*-3 on growth. As both mucin and gum arabic provide fermentable substrate for ruminal bacteria, an additional experiment was performed in which mucin and gum arabic were replaced by equal amounts of starch, cellulose or xylan. No differences in disappearance of 22:6*n*-3 were observed, suggesting that the stimulatory effect of mucin and gum arabic on disappearance of 22:6*n*-3 most probably is not due to provision of an alternative site of adsorption but related to stimulation of bacterial growth. A relatively high proportion of 22:6*n*-3 can be reduced to 22:0 provided the initial concentration is low.

INTRODUCTION

Upon entering the rumen, lipids are extensively hydrolysed and hydrogenated, causing marked differences in fatty acid (FA) profile between the diet and digesta leaving the rumen. The major biohydrogenation pathways of the main 18-carbon PUFA (18:2*n*-6 and 18:3*n*-3), and some of the factors influencing the formation of specific intermediates have been documented (Jenkins *et al*., 2008; Shingfield *et al*., 2013). Much of this information is based on analysing the products formed during incubations of labelled or unlabelled *cis*-9 18:1, 18:2*n*-6 and 18:3*n*-3 with pure or mixed cultures of ruminal bacteria. Even though the long-chain polyunsaturated FA (PUFA), 20:5*n*-3 and 22:6*n*-3 are known to be hydrogenated extensively (Fievez *et al*., 2007), the metabolic pathways responsible and intermediates formed are not well characterised (Jenkins *et al*., 2008). Based on a detailed analysis of omasal digesta of cows fed fish oil, biohydrogenation of 20:5*n*-3 and 22:6*n*-3 were proposed to involve an initial reduction of the double bond closest to the carboxyl group (Kairenius *et al*., 2011). One of the major limitations in identifying the main biohydrogenation pathways is the scarcity of bacterial isolates capable of metabolizing 20:5*n*-3 or 22:6*n*-3. Addition of 22:6*n*-3 is known to inhibit the growth and activity of *B. fibrisolvens* and *B. proteoclasticus* (Maia *et al*., 2007), the major bacterial species capable of biohydrogenation. The antibacterial property of unsaturated free FA is thought to involve alterations in cell membrane function (Desbois and Smith, 2010). It therefore follows that the inhibitory effects of unsaturated FA on bacterial growth may be lessened by minimising the interaction between free FA and bacteria. The present study tested the hypothesis that the addition of adsorbant decreases the interaction of free FA with rumen bacteria. A

total of six experiments were performed. Two experiments were established based on incubations of 22:6*n*-3 with mixed rumen bacteria without or with one of four adsorbants (bentonite, silicic acid, mucin and gum arabic) and involved the measurement of the distribution of 22:6*n*-3 in incubation contents and disappearance of 22:6*n*-3 over a 24-h period. Based on their capacity to interact with free FA, bentonite (Palatsi *et al*., 2012), silicic acid (Proctor and Palaniappan, 1990), mucin (De Weirdt *et al*., 2013) and gum arabic (Xiang *et al*., 2015) were used in the current experiment. As both mucin and gum arabic provide fermentable substrate for rumen bacteria, two additional experiments were performed to distinguish between 1/ decreasing the interaction of free FA and bacteria and 2/ stimulation of bacterial growth. In addition, we evaluated the effect of gum arabic on growth of *B. fibrisolvens* and *B. proteoclasticus*. A final experiment was performed to evaluate differences in the rate of disappearance between 22:6*n*-3 and 18:2*n*-6.

MATERIALS AND METHODS

In vitro incubations with ruminal fluid

All experimental procedures were approved by the ethical commission of the Institute of Agricultural and Fisheries Research, Belgium (ILVO, approval number EC 2009/ 114). Batch *in vitro* incubations were established using rumen contents collected from adult sheep according to Vlaeminck *et al*. (2014). Rumen contents were obtained from three mature wethers fitted with ruminal cannulae fed grass hay *ad libitum* and a grain based concentrate (200 g/day) twice daily at 0900 and 1700-h to

meet their maintenance requirements (Van Der Meer, 1985). Approximately 0.5 L of ruminal digesta was collected from each animal just before the morning feeding. The rumen fluid was filtered through a sieve with a pore size of 1 mm under a stream of CO_2 at 39°C. Donor fluid from each animal were kept separate except for experiment 1 where donor rumen fluid was combined. Rumen inocula was diluted (1:4, v/v) in bicarbonate/phosphate buffer containing a mixture of fermentable substrates (0.21 g glucose, 0.21 g cellobiose, 0.19 g xylose, 0.19 g arabinose, 1.3 g acid casein hydrolysate, 1.3 g peptone and 1.3 g yeast extract per liter of distilled water (Vlaeminck *et al*., 2014)). Diluted buffered rumen fluid (25 mL for experiment 1, 50 mL for experiment 2 and 500 mL for experiment 3) was added to incubation flasks (120 mL flasks for experiment 1 and 2 and 1 L flasks for experiment 3) containing none (control) or one of four adsorbants and 22:6*n*-3 (Nu-check-Prep., Elysian, MN, USA) dissolved in ethanol (20 mg/mL) according to the experimental design (cfr below). On the day before inoculation, adsorbents were added to the incubation flasks. On the day of inoculation, the 22:6*n*-3 solution was added after which the flasks were closed. After flushing with CO_2 during five cycles of 900 mbar underpressure and 600 mbar overpressure, diluted buffered rumen fluid was added. Diluted buffered rumen fluid was added between 30 and 45 min after adding the 22:6*n*-3 solution, allowing 22:6*n*-3 to interact with the adsorbant before inoculum addition. Before incubation flasks were placed at 39°C, overpressure was released. Flasks were maintained under anaerobic conditions at 39°C, with intermittent shaking in a batch culture incubator (Edmund Bühler GmbH, Hechingen, Germany). At the end of each incubation period, the flasks were removed from the incubator and the reaction was stopped by cooling the flasks in an ice bath. Sub-samples were

collected for analysis of volatile fatty acids (VFA: 2 mL) and long-chain FA (LCFA: 5 mL). All treatment combinations were performed in duplicate (technical replicates).

Experiment 1 was performed as a 9 × 3 factorial experiment in a randomized block design to examine the effect of four adsorbants (mucin, gum arabic, bentonite and silicic acid) at two different concentrations (1 and 5 mg/mL) plus a control treatment on the disappearance and metabolism of 22:6*n*-3 added to incubation flasks at three concentrations (0.02, 0.06 and 0.10 mg/mL corresponding to 25, 75 and 125 µL ethanol solution). Each flasks received the same amount of ethanol. Statistical replicates were obtained by using a mixed inoculum of three wethers in three different runs on separate days. After incubation for 24-h, composition of the gas phase (Hassim *et al*., 2010) and pH was measured (Hanna Instruments, Temse, Belgium) and culture contents were sampled for analysis of VFA and LCFA.

Experiment 2 was a 7 × 3 factorial experiment in randomized block design. Cultures containing 0.06 mg/mL of 22:6*n*-3 (corresponding to 150 µL ethanol solution) were incubated up to 48-h (6, 24 and 48-h) to evaluate the disappearance of 22:6*n*-3 in the presence of gum arabic, mucin, cellulose, xylan or starch (5 mg/mL). Two additional treatments were included in the design: a treatment containing no gum arabic, mucin, cellulose, xylan or starch (i.e. only the substrate present in the buffer solution was provided) and a treatment without additional substrate nor carbohydrates in the buffer solution (i.e. removal of glucose, cellobiose, xylose and arabinose from the buffer solution). Statistical replicates were obtained by using inocula from three donor animals. Samples were collected after 6, 24 and 48-h of incubation by removing temporarily the flasks from the incubator and taking 10 mL of incubation fluid using a

syringe. After pH was measured, culture contents were sampled for analysis of VFA and LCFA.

In experiment 3, a single-factor repeated-measures design was used to compare rate of disappearance of 18:2*n*-6 and 22:6*n*-3. Three fermenters (1 L) were used, all containing gum arabic (5 mg/mL), 18:2*n*-6 (0.1 mg/mL, corresponding to 1000 µL ethanol solution prepared by dissolving 18:2*n*-6 (Nu-check-Prep) in ethanol (50 mg/mL)) and 22:6*n*-3 (0.02 mg/mL, corresponding to 500 µL ethanol solution), and were inoculated with diluted rumen fluid (500 mL) and incubated for 24-h in a water bath at 39°C. The concentration of 18:2*n*-6 and 22:6*n*-3 were chosen to allow formation of 18:0 and 22:0, when incubated separately. Inocula for each fermenter was obtained from a different sheep (statistical replicate). Culture content was continuously mixed by means of a magnetic stirrer and the headspace continuously flushed with CO_2 to assure anaerobic conditions. Samples were taken after 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 24-h of inoculation for analysis of VFA and LCFA. Samples were pipetted directly from the fermenter (2 mL for VFA and 5 mL for LCFA) through a closable opening in the lid.

Distribution of 22:6*n*-3 in incubation contents

Two experiments were performed to determine the effect of adding an adsorbant or fermentable substrate on the distribution of 22:6*n*-3 between the solid and liquid phase of diluted rumen fluid. Experiments were carried out in polycarbonate tubes containing 10 mL of diluted buffered rumen fluid supplemented with 0.06 mg/mL of 22:6*n*-3. Diluted buffered rumen fluid was prepared as described before. In experiment 4, the effect of four different adsorbents (mucin, gum arabic, bentonite

and silicic acid at 5 mg/mL) on the distribution of 22:6*n*-3 between liquid and solids was determined. In experiment 5, the effect of gum arabic, mucin, cellulose, xylan and starch (5 mg/mL) was evaluated. All treatment combinations were mixed using a multiple vortex. After 30 min (De Weirdt *et al*., 2013), reactions were stopped followed by centrifugation for 10 min at 3400 **g** at 4°C. Supernatant and pellet were stored at -20°C before LCFA analysis. Preliminary experiments showed that gum arabic and mucin were recovered in the liquid phase and bentonite and silicic acid in the solid phase.

Effect of gum arabic on growth of *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus*

In experiment 6*, B. fibrisolvens* D1 (DSM 3071) and *B. proteoclasticus* P18 (Rowett Institute of Nutrition and Health) were grown anaerobically for 48-h at 39°C in medium 704 (DSMZ) or RM02 medium (Kenters *et al*., 2011) in 12.5 × 1.5 cm culture tubes closed with screw caps fitted with butyl rubber septa (Bellco Biotechnology, Vineland, NJ, USA). The medium 704 was modified by omitting the VFA mixture, haemin and glycerol, and by increasing the proportion of rumen fluid to 20% (v/v). Rumen fluid was collected from three mature wethers. Donor animals were the same as those used for *in vitro* incubations with ruminal fluid and were fed the same diet. Rumen fluid was filtered through a sieve with a pore size of 1 mm and then fine particles were removed from the filtrate by centrifugation at 27 000 **g** for 20 min at 4 °C. The supernatant was sterilized by autoclaving for 20 min at 121 °C and stored frozen at -20 °C. The stored rumen fluid was thawed before use and any new precipitates formed were removed by centrifugation at 27 000 **g** for 15 min at 4 °C. Incubations were carried out anaerobically with and without gum arabic (5 mg/mL).

The 22:6*n*-3 solution was prepared by emulsifying 30 mg of 22:6*n*-3 with polyoxyethylene sorbitan and diluting the mixture in 50 mL of distilled water (0.6 mg/mL) and was added to the medium before autoclaving. Several concentrations of 22:6*n*-3 were evaluated (0, 10, 20, 40, 80 mg/L corresponding to 0, 167, 333, 667 and 1333 µL of the 22:6*n*-3 solution, respectively). Amounts of polyoxyethylene sorbitan (0.1 mg/mL) were kept the same in all tubes by adding 1333, 1166, 1000, 666 and 0 µL of the polyoxyethylene sorbitan solution without 22:6*n*-3. At this concentration, polyoxyethylene sorbitan did not negate the growth inhibitory effects of 18:2*n*-6 on growth of *Propionibacterium freudenreichii* (Rainio *et al*., 2001). Inoculum volumes were 5% (v/v) of a fresh culture that was grown in the same media for 12-h. Growth was measured from the increase in optical density at 600 nm (Ultraspec10, Amersham Biosciences corp., Piscataway, NJ, USA) for duplicate cultures incubated on the same day. Each set of culture experiments was repeated on three different days.

Analysis

Volatile fatty acids in 2 mL of culture contents were prepared according to Castro-Montoya et al. (2012). Samples were analyzed using a gas chromatograph (HP 7890A, Agilent Technologies, Diegem, Belgium) equipped with a flame ionization detector and a Supelco Nukol capillary column (30 m x 0.25 mm x 0.25 µm, Sigma-Aldrich, Diegem, Belgium). The temperature program was initially set at 120°C for 0.2 min; increased at 10°C/min up to 180°C and held at 180°C for 3 min; injector temperature: 250 °C; detector temperature 255 °C. For this temperature program, 0.3 µl was injected with a split/splitless ratio of 25:1 using H_2 as carrier gas at 0.8

mL/min. VFA peaks were identified based on their retention times, compared with external standards (acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid and valeric acid, Sigma-Aldrich, Diegem, Belgium). Samples for LCFA (5 mL) were freeze dried and LCFA were extracted and methylated as described by Vlaeminck et al. (2014). Analysis for fatty acid methyl esters was carried out by gas chromatography (GC) (HP7890A, Agilent Technologies) using a SP-2560 column (75m x 0.18 mm ,i.d. x 0.14 µm thickness, Supelco Analytical, Bellefonte, USA) and a flame ionization detector. The temperature program was initially set at 70°C for 2 min, increased at 15°C/min to 150°C, then increased at 1°C/ min up to 165°C, held at 165°C for 12 min, increased at 2°C/min to 170°C, held at 170°C for 5 min, increased at 5°C/min to 210°C, held at 210°C for 20 min, increased at 5°C/min to 220°C and held at 220°C for 15 min. Inlet and detector temperatures were 250 and 255°C, respectively. The split ratio was 50:1. Hydrogen was used as the carrier gas at a flow rate of 1 mL/min. Peaks were identified based on retention time comparisons with a mixture of FA methyl esters (FAME) standards (GLC463, Nu-Check-Prep., Inc., Elysian, MN, USA and conjugated linoleic acid *cis*-9, *trans*-11-18:2 and *trans*-10, *cis*-12-18:2, Larodan Fine Chemicals AB, Malmö, Sweden). Moreover, aliquots of methylated samples were pooled and subsequently fractionated by Ag^{+}-SPE as described by Kramer et al. (2008). FA commercially not available were identified by order of elution based on Shingfield et al. (2006) and Kramer et al. (2008).

The GC run time was chosen as to allow conjugated isomers of 22:6 to be detected, which would be expected to elute after 22:6*n*-3. As no standards of conjugated isomers of 22:6 are commercially available, conjugated isomers of 22:6 were prepared by alkaline treatment according to Association of Official Analytical Chemist (1990) with modifications. A mixture of potassium hydroxide in ethylene glycol 21%

(w/w) was prepared and degassed for 5 min with nitrogen. A total of 10 mg of 22:6*n*-3 was added to a clean 10 mL test tube containing 4 mL of 21% KOH solution. The mixture was degassed with nitrogen gas, capped and allowed to stand for 5 min at 160°C. After cooling to room temperature, the reaction mixture was acidified to below pH 2 with HCl. The conjugated products were extracted twice with 3 mL of hexane. Spectrophotometric readings (Shimadzu UV-1700; Shimadzu Europe, Duisburg, Germany) confirmed the formation of conjugated dienes (at 233 nm), trienes (268 nm), tetraenes (315 nm), pentaenes (345 nm) and hexaenes (375 nm) (Pitt and Morton, 1957). Initially, the GC run time was extended with the oven temperature at 220°C for 120 min instead of 15 min. As no compounds were observed after the 15 min. at 220°C, this temperature program was used in subsequent analysis.

Calculations

The net amount of VFA produced during the 24-h incubation period was calculated by subtracting the amount of VFA in the rumen fluid/buffer mixture present before and after incubation. Biohydrogenation or disappearance of 22:6*n*-3 was calculated based on the proportional loss of 22:6*n*-3 during the incubation period as the difference of the amount of 22:6*n*-3 before and at the end of the incubation divided by the amount before the incubation. Formation of 22:0 was calculated by subtracting the amount of 22:0 in the rumen fluid/buffer before the incubation from the amount at the end of incubations with 22:6*n*-3.

Rate of disappearance of 18:2*n*-6 and 22:6*n*-3 in experiment 3 were estimated according to a first-order exponential model as:

Residual 18:2*n*-6 or 22:6*n*-3 = $a + b \times \exp(-c \times (\text{time} - \text{lag}))$

where *a* is the amount of FA substrate not hydrogenated, *b* is the amount of FA that can potentially disappear during the incubation with ruminal fluid, *c* is the fractional rate of disappearance of fraction *b* (1/h) with a lag time, h. Parameters were fitted to the data using the NLIN procedure of the SAS (version 9.2; SAS Institute Inc., Cary, NC).

Statistical analysis

Analytical duplicates were averaged before statistical analysis. All statistical analyses were completed using the MIXED procedure of SAS (version 9.2). Differences among means at $P < 0.05$ were considered significant. Differences among least square means were evaluated by the Tukey-Kramer multiple comparison test.

Data from experiment 1 were analysed with a model that included the fixed effects of treatment, 22:6*n*-3 concentration and their interaction and random effect of incubation run. Sums of squares were separated into single degree of freedom orthogonal contrasts to evaluate the linear component of adsorbant addition at each 22:6*n*-3 concentration.

For experiments 2 and 3, data were analysed by ANOVA for repeated measures. For experiment 2, the model included the fixed effects of treatment, incubation time and their interaction and random effect of rumen inoculum. The effect of incubation time was evaluated as a repeated measure using the autoregressive heterogeneous (ARH) covariance structure based on the Akaike's information criterion. For

experiment 3, the model included the fixed effect of incubation time and inoculum source as a random effect. The effect of incubation time was evaluated as a repeated measure using the heterogeneous autoregressive covariance structure based on the Akaike's information criterion.

Data from the experiments evaluating the distribution of 22:6*n*-3 between pellet and supernatants (experiment 4 and 5) were analysed with a model that included the fixed effect of treatment and the random effect of biological replicate (i.e. sheep inoculum).

Data from the pure culture experiment were evaluated with a model that included the fixed effect of treatment (without or with gum arabic) and 22:6*n*-3 concentration and their interaction. Incubation run was included as a random effect. The analysis was performed separately for B. *fibrisolvens* and *B. proteoclasticus* for each growth media.

RESULTS

Influence of adsorbant on the distribution and metabolism of 22:6*n*-3

After 30 min of mixing, a greater proportion of 22:6*n*-3 was recovered in the supernatant when mucin or gum arabic were added (0.345, 0.632 and 0.647 for the control, gum arabic and mucin, respectively, SEM = 0.073, $P < 0.05$). Bentonite or silicic acid did not alter the distribution of 22:6*n*-3 between liquid and solids relative to the control (0.345, 0.227 and 0.398 for the control, bentonite and silicic acid, respectively, SEM = 0.073, $P > 0.05$).

In the follow-up experiment, the effect of adsorbants on the biohydrogenation of 22:6*n*-3 over a 24-h batch *in vitro* incubation was evaluated (experiment 1). Irrespective of treatment, metabolism of 22:6*n*-3 when added at a low concentration (0.02 mg/mL) was extensive (Table 1). Increasing the amount of added 22:6*n*-3 resulted in lowered disappearance of 22:6*n*-3. Increasing amount of mucin or gum arabic increased linearly ($P < 0.05$) 22:6*n*-3 disappearance, at initial 22:6*n*-3 concentrations of 0.06 and 0.10 mg/mL. Addition of bentonite or silicic acid did not alter the disappearance of 22:6*n*-3 compared with the control. Formation of 22:0 was observed when the initial concentration of 22:6*n*-3 was 0.02 mg/mL. At this concentration of 22:6*n*-3, increasing amounts of mucin, gum arabic and bentonite linearly increased the formation of 22:0 ($P <0.05$). For incubations containing a higher initial concentration of 22:6*n*-3 trace amounts of 22:0 were detected.

Influence of amount and type of fermentable substrate on metabolism of 22:6*n*-3 and on fermentation

Addition of mucin or gum arabic resulted in a higher proportion of 22:6*n*-3 being recovered in the supernatant (0.386, 0.558 and 0.543 and for the control, gum arabic and mucin, respectively, SEM = 0.037, $P < 0.05$). Starch, cellulose, xylan and the substrate-depleted control did not alter the distribution of 22:6*n*-3 in incubation contents compared with the control (0.429, 0.397, 0.404 and 0.431 for the starch, cellulose, xylan and the depleted control, respectively, SEM = 0.037, $P > 0.05$). Inclusion of carbohydrates in the media resulted in greater disappearance of 22:6*n*-3 (Table 2).

Table 1. The effect of mucin, gum arabic, bentonite and silicic acid on the disappearance of 22:6*n*-3 and formation of 22:0 during 24-h incubations with mixed rumen fluid. Experiment 1.

		Disappearance of 22:6*n*-3 (g/g) [1,2]			Formation of 22:0 (mg/flask) [1,3]		
	Initial 22:6*n*-3 (mg/mL)	0.02	0.06	0.10	0.02	0.06	0.10
Treatment	Amount (mg/mL)						
Control		1.000	0.639	0.433	0.058	0.003	0.001
Mucin	1	1.000	0.693	0.435	0.076	0.003	0.004
	5	1.000	0.874*	0.522	0.101*	0.001	0.002
Gum arabic	1	0.997	0.721	0.441	0.096*	0.003	0.001
	5	0.987	0.911*	0.651*	0.099*	0.004	0.005
Bentonite	1	0.997	0.624	0.418	0.052	0.004	0.003
	5	0.996	0.718	0.461	0.086	0.003	0.002
Silicic acid	1	0.997	0.704	0.399	0.059	0.003	0.003
	5	0.998	0.724	0.439	0.050	0.003	0.003
SEM		0.003	0.083	0.042	0.021	0.004	0.005
P-value [4]	mucin	1.000	<0.001	0.029	0.003	0.066	0.996
	gum arabic	0.002	<0.001	<0.001	0.017	0.818	0.290
	bentonite	0.380	0.091	0.377	0.013	0.644	0.967
	silicic acid	0.773	0.194	0.644	0.453	0.880	0.794

[1] Values represent least square means (n = 3).
[2] Disappearance of 22:6*n*-3 was calculated based on the proportional loss of 22:6*n*-3 during the incubation period.
[3] Formation of 22:0 was calculated by subtracting the amount of 22:0 in the rumen fluid/buffer before the incubation from the amount at the end of incubations with 22:6*n*-3.
[4] Significance of linear components of the response to adsorbant concentration
* Indicates mean is different from the corresponding control value ($P < 0.05$)

Table 2. The effect of substrate on the disappearance of 22:6*n*-3 (g/g) during incubation with mixed rumen bacteria. Experiment 2.[1]

		Incubation time (h)	
Treatment [2]	6	24	48
Control –	0.240	0.499[a]	0.584[a]
Control	0.391	0.725[b]	0.790[b]
Mucin	0.505	0.874[bc]	0.904[bc]
Gum arabic	0.559	0.931[c]	0.957[c]
Starch	0.422	0.772[bc]	0.851[bc]
Cellulose	0.316	0.785[bc]	0.874[bc]
Xylan	0.429	0.888[bc]	0.928[bc]
SEM	0.104	0.085	0.084
P-value	Treatment	<0.001	
	Time	<0.001	
	Treatment × time	0.062	

[a,b,c] Means with different superscript within a column differ (P < 0.05).

[1] The initial 22:6*n*-3 concentration was 0.06 mg/mL. Disappearance of 22:6*n*-3 was calculated based on the proportional loss of 22:6*n*-3 during the incubation period. Values represent least square means (n = 3).

[2] Control – : no carbohydrates present in the media, control: control – with carbohydrate mixture (0.64 mg/mL), mucin, gum arabic, starch, cellulose, xylan: control with 5 mg/mL of the respective components.

Gum arabic resulted in greater (*P* < 0.05) disappearance of 22:6*n*-3 compared with the control. Replacing gum arabic with equal amounts of cellulose, starch or xylan did not alter 22:6*n*-3 disappearance compared with gum arabic or control. Inclusion of carbohydrates in the media resulted in greater VFA production after 24-h incubation (Table 3). Among the different substrates, the production of VFA showed no significant difference except for cellulose whose VFA production was lower (Table 3).

Table 3. The effect of substrate amount and type on apparent production of volatile fatty acids (µmol/flask) during 24-h incubations with mixed rumen fluid. Experiment 2.[1]

Treatment[1]	Acetate	Propionate	Iso-butyrate	Butyrate	Iso-valerate	Valerate	Total VFA
Control -	265^{a}	44^{a}	0.1^{a}	22^{a}	0.8^{a}	9^{a}	341^{a}
Control +	532^{b}	219^{b}	52.7^{c}	136^{b}	79.6^{d}	131^{b}	1164^{b}
Mucin	1381^{d}	525^{c}	81.3^{d}	280^{d}	115.3^{e}	223^{c}	2623^{d}
Gum arabic	1326^{d}	923^{d}	39.7^{b}	193^{bc}	51.2^{bc}	138^{b}	2677^{d}
Starch	1216^{d}	871^{d}	53.2^{c}	254^{d}	62.6^{cd}	131^{b}	2600^{d}
Cellulose	932^{c}	913^{d}	53.0^{c}	192^{bc}	60.3^{cd}	127^{b}	2283^{c}
Xylan	1388^{d}	975^{d}	39.7^{b}	220^{cd}	38.4^{b}	131^{b}	2797^{d}
SEM	47.0	46.6	2.75	22.1	5.11	2.6	64.5
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

^{a,b,c,d,e} indicates mean are different ($P < 0.05$).

[1] The initial 22:6n-3 concentration was 0.06 mg/mL. Values represent least square means (n = 3).

[2] control-: no carbohydrates present in the media, control+: control- with carbohydrate mixture (0.64 mg/mL), mucin, gum arabic, starch, cellulose, xylan: control+ with 5 mg/mL of the respective components.

Metabolism of 18:2*n*-6 in presence of gum arabic and 22:6*n*-3

Initial concentrations of 18:2*n*-6 were found to decline rapidly during incubations of rumen fluid with 0.1 mg 18:2*n*-6/mL and 0.02 mg 22:6*n*-3/mL (Figure 1). Decreases in 18:2*n*-6 were accompanied by the transient accumulation of conjugated 18:2 isomers, mainly *cis*-9, *trans*-11-18:2, which were further hydrogenated to 18:1 isomers, mainly *trans*-11-18:1 (data not shown). Only after 24-h was an increase in 18:0 detected. Disappearance of 22:6*n*-3 occurred at a slower rate compared with 18:2*n*-6 (rate of disappearance 4.277 *v.* 0.973/h for 18:2*n*-6 and 22:6*n*-3, respectively, SEM = 0.209, $P = 0.005$). Isomerization of 18:2*n*-6 occurred at a high rate, whereas metabolism of 22:6*n*-3 was associated with a lag of 1.79 h after addition to the incubation flask (lag time of 0.13 *v.* 1.79 h for 18:2*n*-6 and 22:6*n*-3, respectively, SEM = 0.309, $P = 0.021$). Only after 24-h was 22:0 formed.

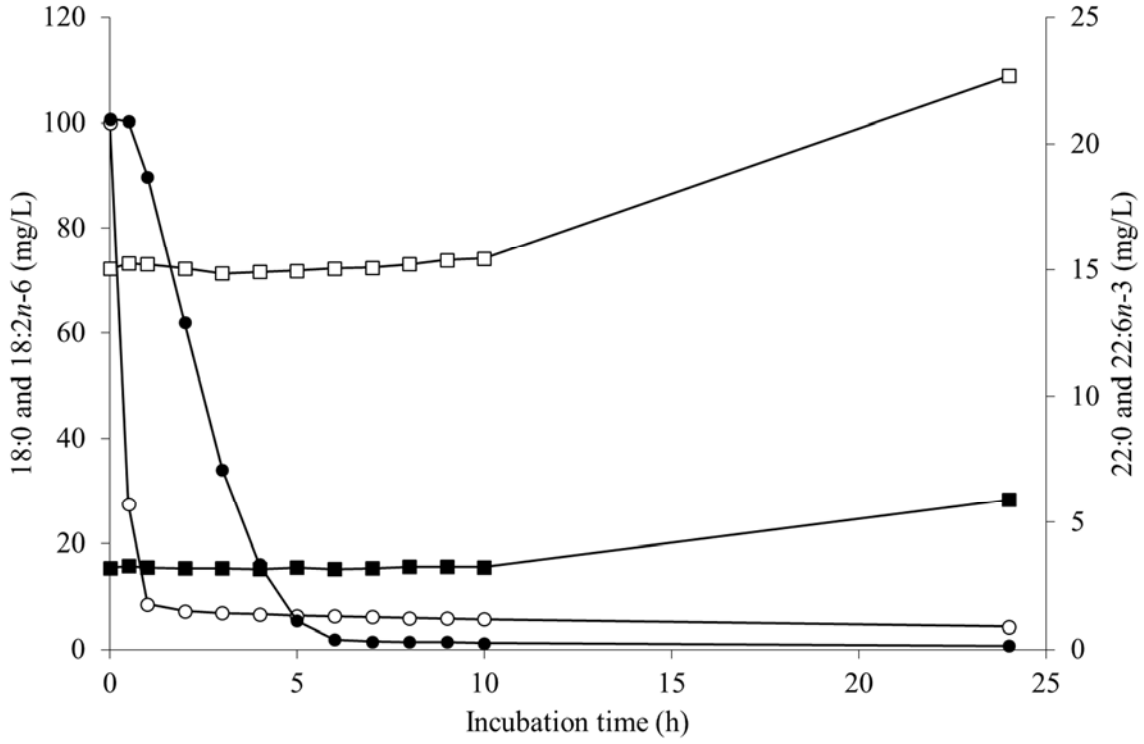

Figure 1. Temporal changes in the concentration of 18:2*n*-6 (open circles), 22:6*n*-3 (filled circles), 18:0 (open squares) and 22:0 (filled squares) during incubation of 18:2*n*-6 (100 mg/L) and 22:6*n*-3 (20 mg/L) with mixed rumen bacteria in presence of gum arabic (5 mg/mL). Values represent least square means (n = 3) (SEM = 1.13, 7.96, 1.13, 0.56 for 18:2*n*-6, 18:0, 22:6*n*-3 and 22:0, respectively). Experiment 3.

Influence of gum arabic and 22:6*n*-3 on growth of *B. fibrisolvens* and *B. proteoclasticus*

Increasing 22:6*n*-3 at inoculation lowered the optical density values measured after 24-h, with the decrease being more pronounced in cultures of B. *proteoclasticus* compared with B. *fibrisolvens*. Addition of gum arabic did not ($P > 0.05$) negate the inhibitory effect of 22:6*n*-3 (Figure 2). Experiment 6.

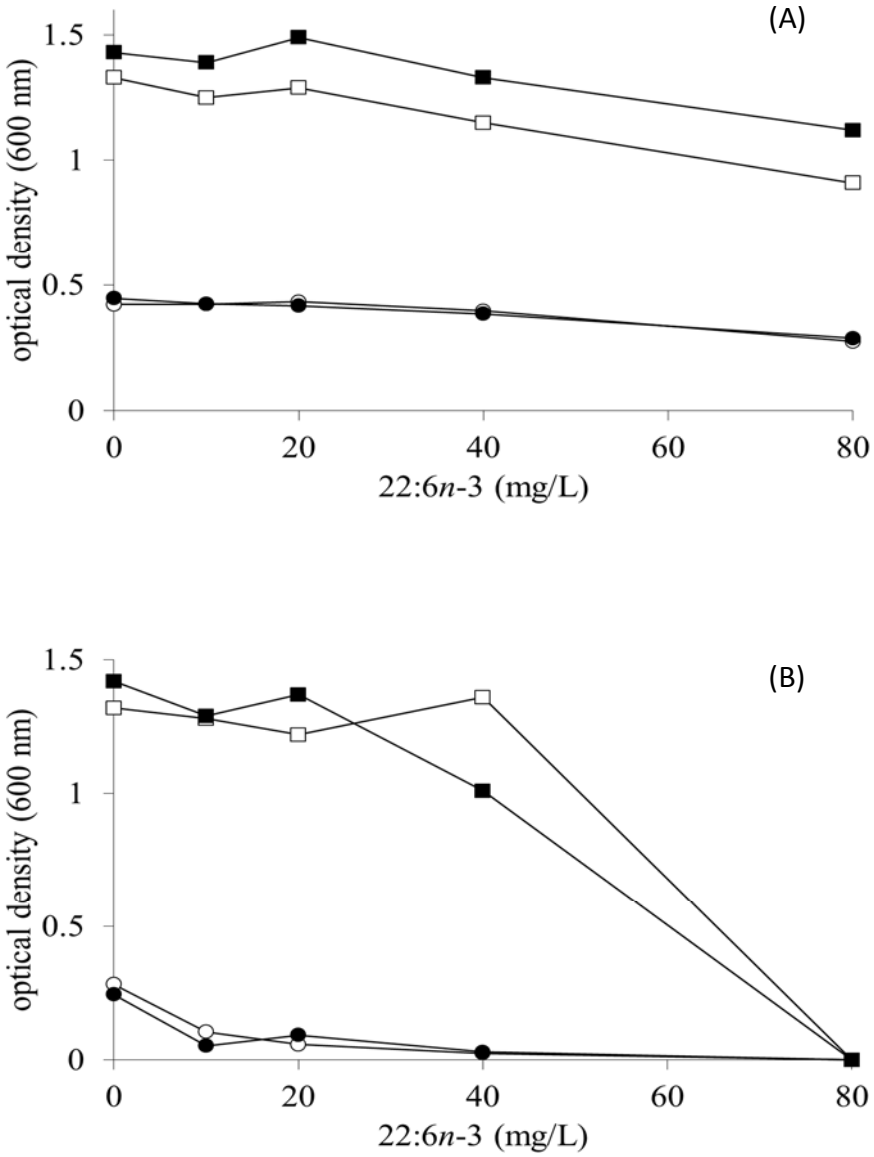

Figure 2. The effect of 22:6*n*-3 concentration without (open symbols) and with (filled symbols) gum arabic on maximum optical density during growth of *B. fibrisolvens* (A) and *B. proteoclasticus* (B) in medium 704 (squares) and RM02 medium (circles). Values represent least square means (n = 3) (SEM = 0.052, 0.058, 0.069, 0.027 for *B. fibrisolvens grown in* medium 704 and RM02 medium and *B. proteoclasticus* grown in medium 704 and RM02 medium, respectively). Experiment 6.

DISCUSSION

Disappearance of 22:6*n*-3

Both 18:2*n*-6 and 18:3*n*-3 are extensively hydrogenated in the rumen. A similar series of isomerization and reduction reactions has been suggested to be involved in the conversion of 22:6*n*-3 to 22:0 by rumen bacteria (Jenkins *et al*., 2008). If the initial step of 22:6*n*-3 biohydrogenation is analogous to that described for 18:2*n*-6 and 18:3*n*-3, initial isomerization of a *cis* double bond would be expected to yield a 22-carbon conjugated product containing 6 double bonds that is subsequently reduced to a 22:5 intermediate (Jenkins *et al*., 2008). However, no 22:6 conjugated FA was detected in omasal digesta of cows fed fish oil suggesting that the biohydrogenation of 22:6*n*-3 may differ from 18-carbon PUFA, by proceeding via the reduction of the *cis* double bond closest to the carboxyl group (Kairenius *et al*., 2011). In the present series of experiments, no accumulation of products eluting in the GC chromatogram with a retention time greater than 22:6*n*-3 was observed (Figure 3), a region where conjugated isomers of 22:6 would be expected to elute with the polar column used in the present study. In order to identify the time window during which conjugated isomers of 22:6 elute, the latter were prepared by an alkaline treatment. Alkali isomerization of 22:6*n*-3 will produce numerous conjugated double bond systems. FA methyl esters of this mixture then have been analysed by GC to identify the retention time window during which these conjugated 22:6 isomers elute. No accumulation of products eluting in the GC chromatogram in this time window were observed (Figure 3).

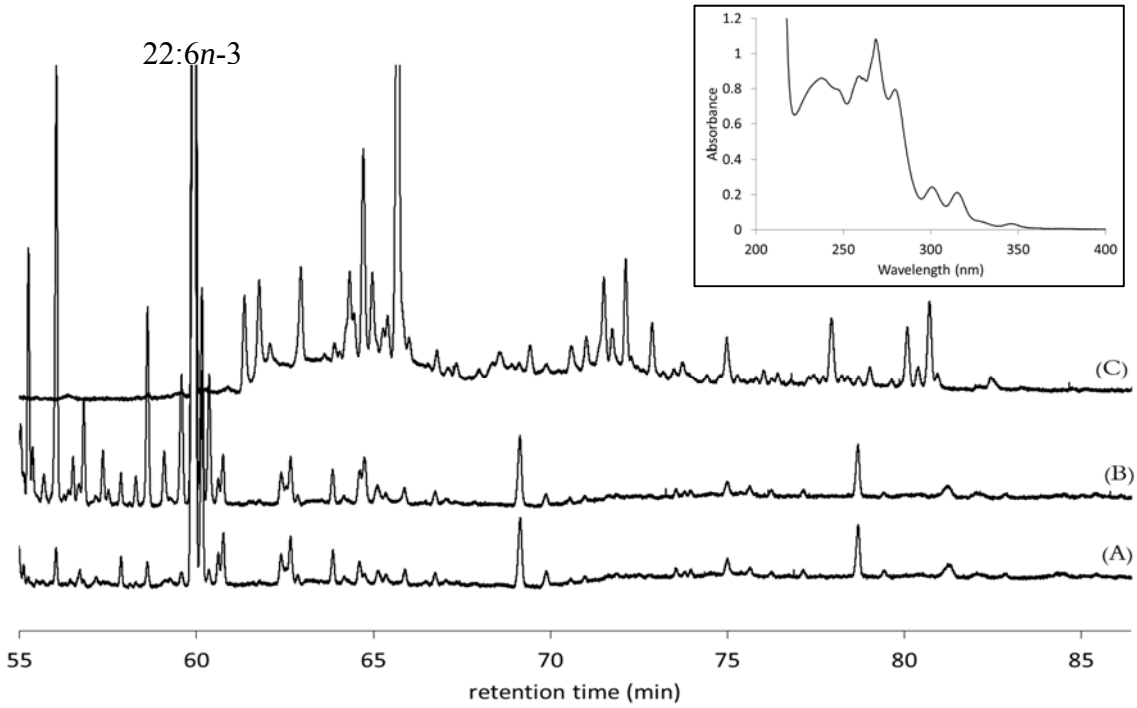

Figure 3. Partial gas chromatogram from (A) 0h incubation and (B) 6 h incubation of mixed rumen bacteria with 22:6*n*-3 (0.02 mg/mL) in presence of gum arabic (5 mg/mL) and (C) conjugated 22:6 prepared by using alkaline treatment. The insert depicts the UV-spectra of conjugated 22:6. Experiment 1.

The lack of accumulation of a conjugated FA might indicate they are transient products which did not accumulate at the time of sampling. This is possible when the rate of formation of this conjugated FA is an order of magnitude smaller than the rate of disappearance. Alternatively, this might also indicate the initial product of 22:6*n*-3 metabolism is not a conjugated FA. Additional investigations based on, for example, GC-MS analysis of the products of 22:6*n*-3 metabolism in the rumen might provide an answer on whether a conjugated FA is the initial product of 22:6*n*-3 metabolism.

In low amounts, disappearance of 22:6*n*-3 was extensive, but biohydrogenation was much lower when higher doses of 22:6*n*-3 were incubated, consistent with previous reports (AbuGhazaleh and Jenkins, 2004b; Aldai *et al*., 2012; Klein and Jenkins, 2011; Vlaeminck *et al*., 2014). In the present study, mucin, gum arabic, bentonite or silicic acid were added to incubation flasks to evaluate if they would influence the disappearance of 22:6*n*-3. We hypothesized these compounds provide an alternative site for adsorption for 22:6*n*-3. As a result, decreasing amounts of 22:6*n*-3 would be available to adhere to bacterial cells and hence adverse effects on bacterial growth and metabolic activity are expected to be reduced. The distribution of 22:6*n*-3 between the pellet and supernatant suggested that addition of bentonite and silicic acid did not largely alter the adsorption of 22:6*n*-3 between liquid and solids. In contrast, addition of gum arabic and mucin resulted in a lesser recovery of 22:6*n*-3 in incubation solids. Our preliminary experiments showed that gum arabic and mucin were recovered in the liquid phase. These findings suggest that both gum arabic and mucin may have provided an alternative to feed particles and bacteria for the adsorption of 22:6*n*-3. At the same time, mucin and gum arabic increased the disappearance of 22:6*n*-3 during the 24-h incubation assay whereas bentonite and silicic acid had no effect. The relation between partitioning and disappearance of 22:6*n*-3 might indicate that the phase distribution plays a critical role in the biohydrogenation of 22:6*n*-3. Consistent with this, mucin was shown to protect *Lactobacillus reuteri* from the inhibitory effects of 18:2*n*-6 by trapping 18:2*n*-6 into the gel-like compartment of mucin lowering the interaction of 18:2*n*-6 with the bacterial cell membrane (De Weirdt *et al*., 2013). Similarly, the growth inhibitory effect of 18:2*n*-6 on *P. freudenreichii* was eliminated by dispersing it in a sufficient

concentration of polyoxyethylene sorbitan monooleate detergent (Rainio *et al*., 2002).

The effect of gum arabic on growth of bacteria in presence of 22:6*n*-3 was evaluated using *B. fibrisolvens* and *B. proteoclasticus*, species known to be involved in the biohydrogenation of 18 carbon PUFA. However, gum arabic did not prevent 22:6*n*-3 inhibiting the growth of either species. Although the contribution of these species to biohydrogenation of 22:6*n*-3 in mixed cultures is unclear (Maia *et al*., 2007), these results might indicate the stimulating effect of mucin and gum arabic on hydrogenation of 22:6*n*-3 in mixed cultures is not directly related to the protection from the growth inhibitory effect of 22:6*n*-3.

Addition of mucin or gum arabic stimulated not only the disappearance of 22:6*n*-3 but also production of VFA during the 24-h batch incubation assay (tables S1 to S3). Gum arabic is a complex mixture of glycoproteins and polysaccharides and mucins are large glycoproteins (Bansil and Turner, 2006). Hence, aside from its colloidal properties, both gum arabic and mucin could potentially serve as fermentable substrate for rumen bacteria. As both VFA production and partitioning of 22:6*n*-3 between the pellet and supernatant were related to disappearance of 22:6*n*-3, an additional experiment was performed in order to differentiate between the protective effect and the substrate effect of gum arabic and mucin. The results illustrate the importance of amount of substrate for extensive disappearance of 22:6*n*-3 but the lack of significant effects between substrates indicates the type of substrate is of lesser importance. The limited amount of fermentable substrate in the depleted control treatment and, to a lesser extent, in the control treatment might be the reason of the decreased biohydrogenation of 22:6*n*-3 in mixed cultures. The availability of

fermentable substrate has been shown to be essential for biohydrogenation as hydrogen needed for biohydrogenation is produced during the fermentation of substrate (Polan *et al.,* 1964). Moreover, the mechanism of adding hydrogen to a double bond is energetically costly because it is NADH dependent (Hunter *et al*., 1976). This suggests that ATP production during the fermentation of substrate and hence the bacterial growth might affect the extent of biohydrogenation. Indeed, biohydrogenation activity of 18:2*n*-6 biohydrogenating bacteria was reported to be higher when the bacteria were growing than when the bacteria were in the stationary growth phase (Wallace *et al*., 2006). This was explained by a limited supply of reducing equivalents once the energy sources in the medium had been depleted as the conversion of CLA to VA is NADH-dependent (Hunter *et al*., 1976; Wallace *et al*., 2006).

Overall, the stimulating effect of mucin and gum arabic most probably is largely due to an increased amount of fermentable substrate and not directly related to the provision of an alternative site for adsorption as originally hypothesized. Combined data from experiment 1 and 2 illustrate disappearance of 22:6*n*-3 was related to apparent VFA production (Figure 4). These data suggest a close association between production of VFA and disappearance of 22:6*n*-3.

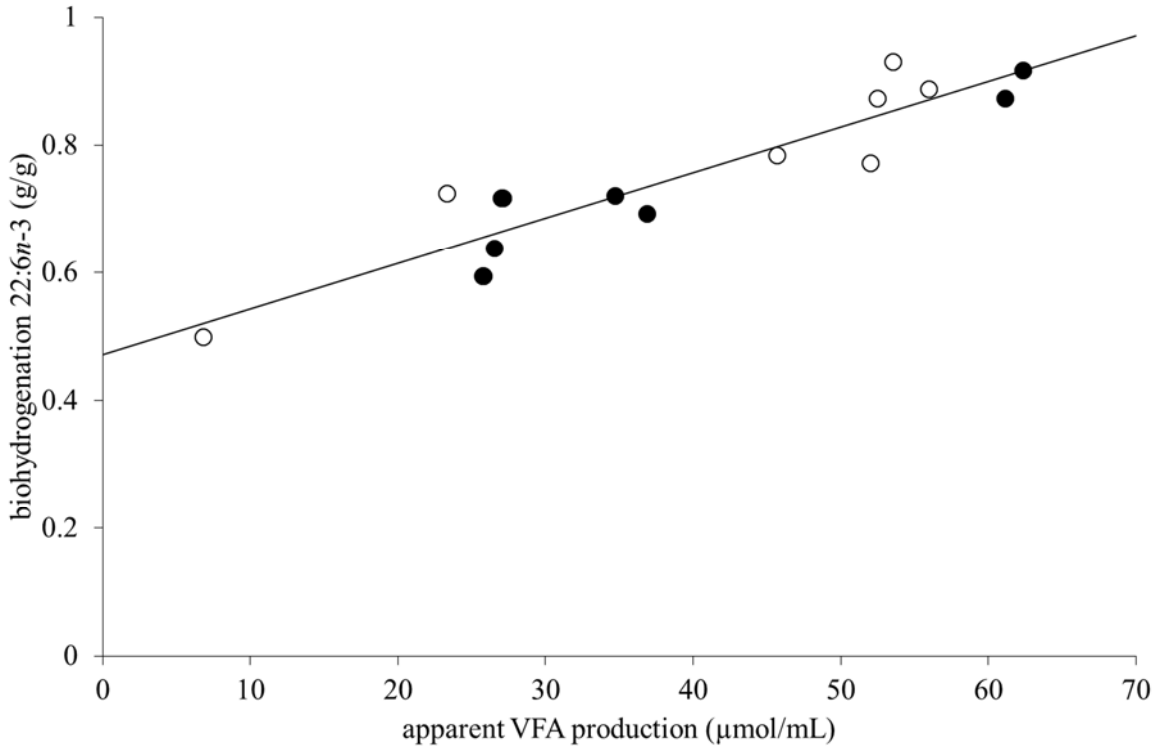

Figure 4. Association of apparent production of volatile fatty acid (VFA) production and disappearance of 22:6*n*-3 during 24-h incubations with 22:6*n*-3 (0.06 mg/mL). Values represent mean values (n = 3) obtained in experiment 1 (filled circles) and experiment 2 (open circles). $Y = 0.472$ (SE = 0.034, $P = 0.046$) $+ 7.14\times10^{-3}$ (SE = 0.804×10^{-3}, $P < 0.001$) $\times X$.

Formation of 22:0

When 22:6*n*-3 was added at a low concentration, up to 0.1 mg 22:0 was formed. At higher initial concentrations of 22:6*n*-3 only trace amounts of 22:0 were formed in agreement with other reports (AbuGhazaleh and Jenkins, 2004b; Aldai *et al*., 2012; Vlaeminck *et al*., 2014). The production of 22:0 from 22:6*n*-3 by ruminal batch cultures has been reported, but only in trace amounts (Klein and Jenkins, 2011) and its production seemed of minor to negligible importance (Shingfield *et al*., 2011,

2012b). When low concentrations were added, up to 20% of the 22:6*n*-3 was recovered as 22:0 indicating that the mixed rumen population is capable of the reduction of all six double bonds. However, the absence of 22:0 formation when initial 22:6*n*-3 increased might indicate that the capacity of rumen microbial communities to reduce double bonds of long-chain PUFA in the rumen is limited (Shingfield *et al*., 2012). The appearance of 22:0 was only observed after long incubation times, indicating that the process is slow. This might be due to the inhibitory effect of 22:6*n*-3 on its biohydrogenation to 22:0, or is merely a reflection of the different steps needed before the substrates of 22:0 formation (i.e. 22:1 isomers) become available.

Previous reports show that the inclusion of 22:6*n*-3 in the diet of ruminants increase the accumulation of 18:1 isomers with little 18:0 formed (Vlaeminck *et al*., 2008), most likely due to the inhibitory effect of 22:6*n*-3 on the bacteria able to convert 18:1 into 18:0 (Boeckaert *et al*., 2008; Kim *et al*., 2008). The formation of the saturated product 22:0 raised the question whether under the same conditions, 18:0 is also formed from 18:2*n*-6. Although the substrates for 18:0 formation (i.e. 18:1 isomers) reached a maximum level after 2 h of incubation, an increase in 18:0 was not detected until the 24-h sampling time. The fact that the inhibitory effect of 22:6*n*-3 on 18:0 formation is reversible, suggests that it is partially driven by 22:6*n*-3 and that 18:0 formation may continue once 22:6*n*-3 concentrations have decreased to a certain threshold. It should be noted that 22:6*n*-3 was absent from 5 h onwards (Figure 1) which might suggest that intermediate products of 22:6*n*-3 metabolism are equally effective in inhibiting the conversion of 18:1 to 18:0. Biohydrogenation is required to lower toxicity and therefore the reduction of products containing more double bonds are a greater priority which explains the lack of 18:1 reduction at time

points where biohydrogenation of 22:6*n*-3 did occur. Once most of the initial 22:6*n*-3 is transformed to less toxic products, both 18:1 and 22:1 isomers are metabolized to their saturated counterparts. This particular sequence of hydrogenation steps might suggest that the bacteria involved in both steps (reduction of 22:6*n*-3 and 18:1) are the same bacteria. Hydrogenation of 18:1 isomers to 18:0 is carried out by *B. proteoclasticus* (Wallace at al., 2006) and, hence, might be responsible for the hydrogenation of 22:6*n*-3.

CONCLUSIONS

The present study tested the hypothesis that the addition of adsorbant provides an alternative site for adsorption of 22:6*n*-3 decreasing the amount available to adhere to bacterial cells and hence lower the adverse effects on growth and metabolic activity. Both mucin and gum arabic increased the disappearance of 22:6*n*-3 during the 24-h *in vitro* assay. The increased disappearance of 22:6*n*-3 observed with mucin and gum arabic was probably due to stimulation of bacterial growth rather than the provision of an alternative site for adsorption. A relatively high proportion of 22:6*n*-3 can be reduced to 22:0 provided the initial concentration is low.

SUPPLEMENTARY INFORMATION CHAPTER 1

Table S1. The effect of mucin, gum arabic, bentonite and silicic acid on the production of CH_4 and apparent production of volatile fatty acids (μmol/flask) during 24-h incubations with mixed rumen fluid and 22:6*n*-3.[1]

Treatment	Amount (mg/mL)	CH_4	Acetate	Propionate	Iso-butyrate	Butyrate	Iso-valerate	Valerate	Total VFA
Control		259	421	93	28.1	65.5	63.3	56.0	733
Mucin	1	306	527	116	29.3	73.7	64.3	64.0	881
	5	467	996	246	38.8	128.9	80.5	104.1	1605
Gum arabic	1	302	533	163	25.9	69.7	57.5	56.0	912
	5	396	915	411	21.4	91.6	43.9	59.4	1545
Bentonite	1	261	407	87	26.7	60.7	59.9	54.1	702
	5	241	413	89	26.6	60.2	58.3	53.7	707
Silicic acid	1	252	388	83	26.7	60.7	58.7	53.7	741
	5	254	463	102	29.0	68.5	63.1	58.0	790
SEM		44.2	69.8	15.4	2.75	11.92	6.80	4.83	104.3
P-value [2]	mucin	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	gum arabic	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.217	<0.001
	bentonite	0.415	0.938	0.858	0.335	0.534	0.170	0.545	0.815
	silicic acid	0.901	0.236	0.284	0.190	0.446	0.630	0.317	0.428

[1] The initial 22:6*n*-3 concentration was 0.02 mg/mL. Values represent least square means (n = 3)

[2] Significance of linear components of the response to adsorbant concentration

Table S2. The effect of mucin, gum arabic, bentonite and silicic acid on production of CH_4 and apparent production of volatile fatty acids (μmol/flask) during 24-h incubations with mixed rumen fluid and initial 22:6*n*-3.[1]

Treatment	Amount (mg/mL)	CH_4	Acetate	Propionate	Iso-butyrate	Butyrate	Iso-valerate	Valerate	Total VFA
Control		183	357	91.5	26.6	58.7	52.3	61.0	663
Mucin	1	250	519	140	30.4	79.4	66.0	75.7	922
	5	389	891	265	38.7	125.5	80.9	112.2	1528
Gum arabic	1	186	469	184	25.2	68.3	55.5	61.8	868
	5	317	868	444	23.7	101.2	51.2	65.8	1558
Bentonite	1	144	348	83.9	26.3	57.7	59.0	59.4	643
	5	164	374	87.8	26.9	60.9	56.9	59.1	677
Silicic acid	1	186	343	88.9	26.4	58.2	58.6	60.3	644
	5	187	364	92.2	27.1	61.9	60.0	60.6	675
SEM		45.5	70.4	15.7	2.71	10.83	6.21	5.26	107.0
P-value [2]	mucin	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	gum arabic	<0.001	<0.001	<0.001	0.015	<0.001	0.020	0.120	<0.001
	bentonite	0.401	0.662	0.935	0.646	0.687	0.774	0.631	0.751
	silicic acid	0.888	0.794	0.911	0.520	0.606	0.732	0.946	0.787

[1] The initial 22:6*n*-3 concentration was 0.06 mg/mL. Values represent least square means (n = 3)

[2] Significance of linear components of the response to adsorbant concentration

Table S3. The effect of mucin, gum arabic, bentonite and silicic acid on production of CH_4 and apparent production of volatile fatty acids (μmol/flask) during 24-h incubations with mixed rumen fluid.[1]

Treatment	Amount (mg/mL)	CH_4	Acetate	Propionate	Iso-butyrate	Butyrate	Iso-valerate	Valerate	Total VFA
Control		148	289	78.4	24.0	45.4	54.6	61.0	564
Mucin	1	191	446	125.4	28.7	62.1	61.5	80.4	821
	5	296	843	256.1	37.4	103.4	78.0	123.2	1465
Gum arabic	1	185	393	162.4	24.3	59.9	52.6	62.8	763
	5	317	790	443.8	23.0	94.5	48.2	65.5	1468
Bentonite	1	143	333	88.4	24.0	51.8	55.9	60.8	623
	5	163	354	88.8	26.5	55.7	59.1	61.4	655
Silicic acid	1	142	303	84.0	25.5	48.8	56.3	61.9	590
	5	140	317	89.6	25.7	53.3	57.2	60.8	613
SEM		43.9	69.2	16.6	2.41	8.20	5.43	7.70	105.2
P-value [2]	mucin	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	gum arabic	<0.001	<0.001	<0.001	0.503	<0.001	0.020	0.547	<0.001
	bentonite	0.529	0.355	0.706	0.132	0.242	0.089	0.947	0.410
	silicic acid	0.827	0.663	0.619	0.461	0.327	0.369	0.936	0.638

[1] The initial 22:6*n*-3 concentration was 0.10 mg/mL. Values represent least square means (n = 3)

[2] Significance of linear components of the response to adsorbant concentration.

Chapter 2: Dilution-to-enrichment in combination with molecular techniques to investigate rumen bacteria involved in 22:6*n*-3 biohydrogenation.

ABSTRACT

Docosahexaenoic acid (22:6*n*-3) is extensively biohydrogenated by rumen microbes residing the rumen. However, knowledge on the microbes involved is limited. In this study, we attempted to identify potential rumen bacterial species involved in the biohydrogenation of 22:6*n*-3 by a dilution-to-enrichment experiment in combination with molecular techniques. Fir this, rumen fluid was serially diluted (100-fold) to produce cultures with different amounts of rumen inocula (dilution ranges from 10^2 to 10^{14}).

The amounts of 22:6*n*-3 after a 48-h incubation period progressively accumulated as the inoculum was more diluted. Both denaturing gradient gel electrophoresis (DGGE) and 16S rRNA amplicon sequencing showed that dilution of the inoculum resulted in different microbial profiles which were accompanied with changes in the residual amount of 22:6n-3. Putative taxonomic identification of DGGE key bands corresponded to *Streptococcus* species and *Selenomonas ruminantium* Y6. However, in pure cultures these species failed to show biohydrogenation. Relative abundance analysis of 16S rRNA amplicon sequencing showed that the phylum *Firmicutes* was most abundant in dilution levels 10^2 to 10^8 (≈50%), but its dominance reduced to 30% in the 10^{10} dilution. *Streptococcaceae*, *Lachnospiracea*, and *Rikenellaceae* were the three major families in this phylum and they were negatively correlated with residual 22:6*n*-3.

INTRODUCTION

Docosahexaenoic acid (22:6*n*-3) is a polyunsaturated fatty acid (PUFA) that has been associated with physiological benefits in humans as well as in ruminants (Baker *et al*., 2016; Mattos *et al*., 2004). Several studies have shown that in ruminants the amount of 22:6n-3 available for absorption in the small intestine can be increased by intake of marine products (e.g. fish oil, marine algae; Franklin *et al*., 1999; Toral *et al*., 2010). However, upon ingestion, 22:6*n*-3 is extensively biohydrogenated in the rumen by rumen microbes which limits the amount of 22:6*n*-3 available for absorption.

Biohydrogenation of PUFA in the rumen is thought to be a detoxification mechanism of the bacteria to escape from the inhibitory effects of PUFA (Maia *et al*., 2010). Several bacteria involved in the biohydrogenation of linoleic and linolenic acid have been identified (Harfoot and Hazlewood, 1997; Wallace *et al*., 2006). In contrast, knowledge of the bacterial species responsible for the biohydrogenation of 22:6*n*-3 is absent. Several attempts to identify bacterial species able to metabolize 22:6*n*-3 failed (Maia *et al*., 2007; Potu *et al*., 2011; Wasowska *et al*., 2006). The high sensitivity of rumen bacteria towards 22:6*n*-3 might be the main reason for this (Maia *et al*., 2007). Furthermore, the toxic effect of 22:6*n*-3 towards several rumen bacteria is related with inoculum size (Vlaeminck *et al.*, 2014), resulting in a rapid decrease in biohydrogenation of 22:6*n*-3 upon reduction of the inoculum size of mixed rumen cultures (Vlaeminck *et al*., 2014). Hence, to allow an inoculum dilution-to-enrichment approach, firstly the possibility was examined to enhance 22:6n-3 biohydrogenation through the addition of autoclaved rumen fluid particles (uncentrifuged-autoclaved rumen fluid; uRF). Indeed, Harfoot *et al*. (1973) showed that addition of rumen fluid

particles stimulated biohydrogenation of FA by ruminal bacteria (Harfoot *et al*., 1973). Accordingly, we hypothesized that the addition of these particles would allow biohydrogenation of 22:6*n*-3 with reduced inoculum size, which was assessed in experiments 1 and 2. Further, an inoculum dilution-to-enrichment technique (experiment 3) was performed in which denaturing gradient gel electrophoresis (DGGE) and 16S rRNA amplicon sequencing were used to investigate the species composition of the diluted cultures. Potential bacteria involved in the biohydrogenation process of 22:6*n*-3 were identified by excising selected DGGE bands and by identification of the relative bacterial composition of the samples showed 22:6*n*-3 disappearance through next generation sequencing. At last, some close relatives of the bacterial species cloned from the DGGE bands were tested for their ability to metabolize 22:6*n*-3 *in vitro* (experiment 4).

MATERIALS AND METHODS

Rumen Inocula

Rumen inocula was obtained from three mature wethers fitted with a ruminal cannula and fed a hay/commercial pelleted grain based concentrate diet twice a day according to their maintenance requirements. These animals are maintained by the Institute for Agricultural and Fisheries Research (ILVO, Belgium) and fistulation was approved by the ethical commission (File number 241, 2014) of the ILVO.

Uncentrifuged-Autoclaved Rumen Fluid (uRF)

To obtain uncentrifuged-autoclaved rumen fluid (uRF), rumen fluid collected from cannulated wethers was filtered through a sieve (pore size 1mm) and autoclaved for 20 min at 121°C. For experiment 1 (see below), rumen fluid from three sheep was combined, autoclaved and stored at -20 °C until use. In experiment 2 (see below), rumen fluid from the three individual sheep was kept separate throughout the experiment. In this experiment, uRF was used on the day of preparation. For experiment 3, uRF from one sheep was used to prepare the growth media for the enrichment cultures.

In vitro incubations

Two experiments (Experiment 1 and experiment 2) were carried out to evaluate the effect of uRF on the disappearance of 22:6*n*-3. Incubations were performed as described by Vlaeminck et al. (2014). Approximately 0.5 L of ruminal digesta was collected from each animal just before the morning feeding. Rumen inoculum was obtained by filtering the digesta samples through a sieve (pore size 1 mm) under continuous CO_2 flushing at 39 °C. Rumen inocula were kept separate per animal resulting in 3 biological replicates per treatment.

A bicarbonate/phosphate buffer that contained the fermentable substrate (Vlaeminck *et al*., 2014) was used in experiments 1 and 2. The incubations were performed in Pyrex tubes for 24-h under intermittent shaking at 39 °C in a batch culture incubator (Edmund Bühler GmbH, Hechingen, Germany). Each tube contained inoculum, buffer, water and/or uncentrifuged-autoclaved rumen fluid (uRF). Docosahexaenoic acid (U-84-A, Nu-Check-Prep., Elysian, MN, USA) was dissolved in ethanol and

added to all the tubes according to the experimental requirements. At the end of the incubation period, the reactions were stopped by cooling the tube in an ice bath and culture pH was recorded immediately (Hanna Instruments, Temse, Belgium). Samples were collected for volatile fatty acids (VFA) and long chain fatty acids (LCFA) analysis. Gas chromatographic methods for the determination of VFA was performed as described by Gadeyne et al. (2016) and LCFA analysis was performed as described by Vlaeminck et al. (2014).

Experiment 1

In experiment 1, we evaluated disappearance of 22:6*n*-3 as affected by the addition of uRF to cultures containing different amounts of rumen inoculum. The total volume of the incubation fluid was 10 mL/tube. The proportion of buffer was 0.5 (v/v) of total incubation fluid for all cultures. Proportions of rumen inoculum were 0.05, 0.2, 0.35 and 0.50 of the total incubation volume and either water or uRF (0.45, 0.30, 0.15 or 0.0; v/v) was added to the cultures. The initial amount of 22:6*n*-3 was 100 µg/mL.

Experiment 2

As the design of experiment 1 did not allow to quantitatively evaluate the effect of amount of uRF independently from the proportion of rumen inoculum, we examined the effect of mixtures containing different proportions of inoculum, uRF and water on the disappearance of 22:6*n*-3 according to a constrained simplex lattice design. The total volume of incubation fluid was 10 mL/tube of which the amount of buffer was equal for all treatments (0.5, v/v). In addition, all treatments were set to include at

least 0.05 (v/v) rumen inoculum. The remaining part of the incubation fluid (i.e. 0.45, v/v) contained rumen inoculum, uRF and/or water according to a simplex lattice design for a three component mixture. Ten experimental treatments (Table 1) showing different levels of ingredient combinations were generated with rumen inoculum, uRF and water ranging from 0.05 - 0.50, 0 - 0.45 and 0 - 0.45 (v/v), respectively. For treatments in which the culture tube contained both uRF and rumen inoculum, both compounds originated from the same donor animal. The initial concentration of 22:6*n*-3 was 60 or 100 µg/mL.

Table 1. Composition of the mixtures of rumen inoculum, uncentrifuged autoclaved rumen fluid (uRF) and water used to evaluate their effect on residual 22:6*n*-3 during 24-h batch incubations. (experiment 2, n=3).

Treatment	Coded values [a]			Actual composition values (v/v/v)[b]		
	Rumen inoculum	uRF	water	Rumen inoculum	uRF	water
1	0	0	1	0.05	0	0.45
2	0	0.333	0.666	0.05	0.15	0.30
3	0	0.666	0.333	0.05	0.30	0.15
4	0	1	0	0.05	0.45	0
5	0.333	0	0.666	0.20	0	0.30
6	0.333	0.333	0.333	0.20	0.15	0.15
7	0.333	0.666	0	0.20	0.30	0
8	0.666	0	0.333	0.35	0	0.15
9	0.666	0.333	0	0.35	0.15	0
10	1	0	0	0.50	0	0

[a] the coded values are used to fit the mixture model.
[b] for all treatments, the amount of buffer in the incubation media was identical and represented half of the incubation media.

Dilution-to-enrichment experiment (experiment 3)

Approximately 500 mL of ruminal digesta was collected from three sheep before the morning feeding, pooled mixed with a kitchen blender and strained through four layers of cheesecloth. Rumen fluid was serially diluted (100-fold) to produce cultures with different amounts of rumen inocula (dilution ranges from 10^2 to 10^{14}). Serial dilutions were prepared in growth medium 330 (DSMZ rumen bacteria medium). This medium was slightly modified: volatile fatty acids mixture, haemin and glycerol were omitted and L-Cysteine was used as the only reducing agent (0.5 g/L). Initial dilution tubes contained uRF (0.5 v/v). Dilutions were inoculated (1 mL) in Hungate tubes containing growth medium 330 (9 mL) supplemented with 22:6*n*-3 (10 µg/mL). Growth medium was the same as used for preparation of serial dilutions with the exception that centrifuged rumen fluid was replaced by uRF (0.5 v/v). The required amounts of 22:6*n*-3 were added as a FA solution (explained below) individually to each tube before autoclaving the Hungate tubes containing growth media. After inoculation, tubes were incubated in triplicate at 39 °C with intermittent shaking in a batch culture incubator, and incubation was stopped at 24-h, 48-h or 144-h. All mixing, dilution and inoculation were carried out under CO_2 environment. The headspace gas used for this incubation was H_2. pH was measured and culture contents were sampled for VFA, LCFA and microbial analysis.

Fatty acid solution

Fatty acid solution was prepared by dispersing 20 mg of 22:6*n*-3 (Nu-check-Prep., Elysian, MN, USA) in 1.5 mL of a 0.06 M Tween-80 (Sigma Aldrich, St Louis, MO) solution. Then 0.15 mL of 3 M NaOH was added to obtain a clear solution. This

solution was diluted with distilled water to achieve a final 22:6*n*-3 concentration of 1 mg/mL.

Total DNA extraction

Bacterial community composition was determined by the DGGE technique and 16S rRNA amplicon sequencing. Only the samples collected at 48-h incubation were used for this purpose. Total genomic DNA was extracted from samples using a bead beating and column purification (QIAamp DNA stool mini kit, Qiagen, Valencia, CA) method (Yu and Morrison., 2004). The yield and purity of the extracted DNA was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and extracted DNA was stored at -20 °C. The primers used in this study are listed in Table S1.

Denaturing Gradient Gel Electrophoresis (DGGE)

All the PCR for DGGE were performed in an Applied Biosystems 2720 thermal cycler (ThermoFisher Scientific, Waltham, MA, USA) and the resultant PCR products were analysed using agarose gel (2.0% [w/v]) electrophoresis. The bacterial-specific primers 338F-GC and 518R (Table S1) were used to amplify a 180-bp product covering the V2-V3 region of the 16S rRNA gene for DGGE. The PCR (50 µl) contained 5 µl of 10× reaction buffer without $MgCl_2$ (Thermo Scientific), 3 µl $MgCl_2$ (25 mM, Thermo Scientific), 1 µM of each primer (Sigma Aldrich, St Louis, MO), 200 µM of each dNTP (Thermo Scientific), 1.25 U of Taq DNA polymerase (Thermo Scientific), 0.125 µl of bovine serum albumin (20 mg/mL, Thermo Scientific), 25 ng of

template DNA and DNA-free water. The amplification was performed using the following cycling parameters: an initial denaturation for 4 min at 95 °C; 25 cycles each of denaturation for 30 s at 95 °C, annealing for 30s at 55 °C and primer extension for 1 min at 72 °C; and a final extension of 10 min at 72 °C.

DGGE was performed using the Bio-Rad D gene system (Bio-Rad, Hercules, CA). polyacrylamide gels (8% w/v) were prepared and electrophoresed with 1× TAE buffer (40 mM tris (hydroxymethyl) aminomethane, 65 mM acetic acid and 1 mM EDTA, adjusted to pH 8.0). The gels contained a gradient of denaturant that increased in the direction of electrophoresis, with a 45- 60% v/v gradient. The 100% denaturant solution contained 40% v/v formamide and 7.0 M urea. The bacterial 16S rRNA PCR products were loaded into each well. Electrophoresis was performed at 38 V (constant voltage) and 60 °C (constant temperature) for 16 h. The gels were stained with 8 µl GelRed nucleic acid gel stain (10,000X, Biotium, Fremont, CA, USA) in 200 mL of 1× TAE buffer for 20 min with gentle shaking. The gels were viewed using UV transillumination and photographed using Chemidoc MP imaging system (Bio-Rad, Hercules, CA). A homemade reference standard of different PCR fragments generated using the primers 338F-GC and 518r from DNA extracted from pure cultures of 6 bacteria (*Spironucleus salmonicida*, *Vibrio anguillarum* strain NB10, *Streptococcus agalactiae*, *Vibrio harveyi*, *Vibrio anguillarum* strain VQ775, *B. proteoclasticus* strain P18*)* was loaded in each gel.

To extract DNA from DGGE bands, selected bands were cut from the gel using a clean, sharp scalpel and transferred to a 1.5-mL microcentrifuge tube. DNA was eluted from polyacrylamide gel slices according to the methods described by Etokebe and Spurkland, (2000). Briefly, gel slices were washed by incubation in 50 µl of water

for 15 min at room temperature. The water was then removed and discarded. DNA was recovered by the addition of 50 µl of UV-treated water to each tube, followed by incubation overnight at 4 °C in 1.5-mL tubes. The tubes were vortexed for 5 s, centrifuged for 1 min at 10,000 **g** and the supernatant was transferred to clean tubes. Another DGGE were performed to confirm the purity and position of migration of cut bands. The DNA fragments in the supernatants were ligated into a plasmid vector (pGEM-T vector systems; Promega, Madison, WI, USA) and either directly sequenced after amplification with the primers T7 (TAA TAC GAC TCA CTA TAG GG) and 518r (Table S1) or cloned into competent *Escherichia coli* TOP-10 cells. Sequencing was performed at LGC Genomics GmbH (Germany).

Analysis of DGGE bands

DGGE fingerprints were analysed with the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium). Each profile, consisting of a digitized image from the gel and representing one lane containing one separated PCR product, was normalized relative to the bands in the lane containing the reference standard. Similarities between the fingerprints were quantified using the Pearson correlation coefficient between each pair of profiles, and these values were represented graphically using the Unweighted Pair Group Method of Arithmetic Averages (UPGMA) method.

Phylogenetic analyses

Phylogenetic analysis was implemented for sequences of DGGE bands in MEGA 7.0 (Kumar *et al*., 2016). Nucleotide sequences were globally aligned with CLUSALW using the IUB DNA matrix and the transition weight of 0.5 phylogenetic trees were constructed using the neighbour-joining inference method (Saitou and Nei, 1987) with the Jukes-Canter substitution model (Jukes and Cantor, 1969) assuming uniform rates among sites and using the completed deletion of gaps option.

Nucleotide sequence accession numbers

Nucleotide sequences of DGGE bands of this study have been deposited in GenBank and their accession numbers are given in Table 4.

Bacterial 16S rRNA amplicon sequencing

Bacterial 16S rRNA amplicon sequencing (V3–V4 region) was done on all 21 samples (n = 3 for each dilution). Preparation of the amplicons barcoded library was based on the Illumina 16S metagenomic sequencing library preparation protocol (https://support.illumina.com) and performed by Macrogen (Korea). The sequencing was performed using Illumina MiSeq V3-technology (2 × 300bp) by Macrogen (Korea).

The amplicon sequencing dataset was demultiplexed by the sequence provider and barcodes were clipped off. Primer sequences were removed using Trimmomatic v0.32 (Bolger *et al*., 2014). The forward and reverse reads were merged using a minimum overlap length of 120 bp, a minimum and maximum resulting length of 400

and 450 bp and a quality threshold of 30 with a minimum length of 200 bp after trimming, using PEAR 0.9.8 (Zhang *et al*., 2014).

Quality filtering was done using 'fastq_filter' with a maximum expected error of 3. Sequences of all samples that needed to be compared were concatenated, dereplicated ('derep fulllength') and sorted by size ('sortbysize'). Uparse ('cluster_otus') was used to cluster the reads into operational taxonomic units (OTUs) at 97% identity level (Edgar, 2013). Chimeras were removed using Uchime ('uchime_ref') with the RDP Gold database as a reference (Edgar *et al*., 2011). Finally, sequences of individual samples were mapped back to the representative OTUs and converted to an OTU table (McDonald *et al*., 2012) resulting OTU tables were annotated with the QIIME software package (v1.9.1) (Caporaso *et al*., 2010a). Representative OTU sequences were aligned to the Greengenes 97% core OTU set (v13_8) (DeSantis *et al*., 2006) , with a minimum percent identity of 97% using the PyNast algorithm (Caporaso *et al*., 2010b) with QIIME default parameters.

Quality filtering resulted in an average of 68460 ± 8340 reads per sample with an average read length of 417 bp. Rarefaction analyses (Figure S4) were done using the QIIME software package indicating that a sequencing depth is sufficient to analyse the bacterial communities in all samples. Shannon-Wiener diversity, Simpson diversity indices and observed richness were calculated with the phyloseq package in R (McMurdie and Holmes, 2013). For subsequent data analysis, only OTUs representing at least 0.1% of the total community in at least one sample were retained. Differences between dilution level (inoculum-dilution) were analysed by PERMANOVA, using the adonis function in Qiime. Relative abundance tables were generated at the genus and family level and checked for their significant differences

among different inoculum-dilution. A heatmap of the relative abundance at family level was generated using the R-package gplots and the heatmap.2 function, using Manhattan distances and UPGMA (unweighted pair group method with arithmetic mean) for hierarchical clustering.

Pure culture experiment (experiment 4)

Based on the findings of the dilution-to-enrichment experiment and DGGE, 2 bacterial species were selected for pure culture experiments: *Selenomonas ruminantium* (*S. ruminatium lactilytica* DSM 2872 and *S. ruminatium ruminatium* DSM 2150) and *Streptococcus gallolyticus* (DSM 16831). These bacteria were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and cultured according to the DSMZ specified culture conditions.

The growth medium used for the pure culture experiments was the same media used for experiment 3 but contained 20% centrifuged-autoclaved rumen fluid. *In vitro* incubations were carried out anaerobically at 39 °C under continuous shaking in Hungate-type tubes containing 9.5 mL of medium with supplemented 22:6*n*-3. We used 3 different concentrations of 22:6*n*-3 in our experiments: 5, 10 and 20 µg/mL incubation medium. 22:6*n*-3 was added as a fatty acid solution (as mentioned in experiment 3) before autoclaving to Hungate tubes individually. Inoculum volumes were 5% (v/v) of a fresh culture that was grown for 24-h. Growth was determined by measuring the culture's optical density (OD) at 600 nm (Ultraspec 10, Amersham Biosciences corp., Piscataway, NJ, USA). At the end of the experiment (24-h), the incubations were stopped by placing the tubes in ice water. pH was measured and

culture contents were samples for VFA and LCFA analysis. Three replicates were used for each concentration and samples were collected at 0-h and 24-h.

Statistical analysis

All statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC). Differences among means with $P < 0.05$ were considered to be statistically significant. Differences among least square means were evaluated using a multiple comparison test following Tukey-Kramer method for adjustment.

Data from experiment 1 were treated to quantify the effect of uRF on the residual amount of 22:6*n*-3 after 24-h incubation according to the following model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \xi$$

where β_0 is the overall intercept; β_1, β_2 and β_3 are the regression coefficients, X_1 is the inoculum size, X_2 is the presence of uRF (present or absent) and ξ is the residual error. Biological replicate and the interaction of replicate with inoculum, uRF and their interaction were included as a random effect. The Satterthwaite method was used to calculate the degrees of freedom.

For experiment 2, data were analysed according to a simplex lattice mixture design to evaluate the effect of inoculum, uRF and water on the residual amount of 22:6*n*-3 after a 24-h incubation period according to the canonical form of the mixture model:

$$Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \xi$$

where β_1, β_2, β_3, β_{12}, β_{13}, β_{23} are regression coefficients, X_1 is the inoculum size, X_2 = is the amount of uRF, X_3 is the amount of water and ξ is the residual error. The model included the random effect of the interaction of replicate with inoculum, uRF,

water, and their interaction. For this analysis, the coded values (Table 1) are used to fit the model following the standard procedures according to lattice design (Cornelius, 2004). The range for each component in the mixture is set to vary from 0 to 1, to avoid collinearity problems during statistical analysis.

For experiment 3, least squares mean in the GLM procedure using dilution level or incubation time as factors was used to test dilution level, incubation time and dilution level × incubation time. Statistical analysis (ANOVA) was conducted in SAS version 9.4 (SAS Institute Inc., Cary, NC) to identify differences of the bacterial composition between dilutions. Relative abundance data were used to estimate the correlation of individual family-level taxa with residual 22:6*n*-3 (10^2 to 10^{10}) using Spearman's Rank. A value of $p \leq 0.05$ was regarded as statistically significant. Principal coordinate analysis and was conducted in QIIME to identify community-wide differences among different level of dilution-enrichment tubes.

RESULTS

Effect of uRF on the disappearance of 22:6*n*-3 (experiment 1)

An increase in inoculum size decreased the residual amount of 22:6*n*-3 ($P = 0.048$), but the effect was dependent on the presence of uRF as indicated by the significant interaction term (110.6, $P = 0.015$) (Table 2). When uRF was present, changes in inoculum size only marginally affected the residual amount of 22:6*n*-3 (Figure S1). In contrast, when uRF was not present, the residual amount of 22:6*n*-3 linearly increased from 25.8 ± 19.9 μg/mL when inoculum represented half of the incubation

liquid to 80.9 ± 4.4 µg/mL when only 0.5 mL of inoculum was present in the 10 mL incubation liquid (Figure S1).

Table 2. Model to determine the residual amount of 22:6*n*-3 (µg/mL) after 24-h incubation of rumen inoculum (0.05, 0.20, 0.35 or 0.50, v/v) with or without addition of uncentrifuged-autoclaved rumen fluid (uRF) (n=3) (experiment 1)

Explanatory variable/level		*β*[1]	SE (*β*)[2]	P-value
Intercept		86.70	3.12	0.001
Inoculum		-123.31	28.07	0.048
aRF	without	-	-	-
	with	-57.41	4.49	0.006
Inoculum × aRF	without	-	-	-
	with	110.59	13.80	0.015

[1] Coefficient from model.

[2] Standard error of the *β* value.

Effect of different proportions of uRF and inoculum on the disappearance of 22:6*n*-3 (experiment 2)

In Experiment 2, we used a simplex lattice design to evaluate the effect of different proportions of uRF and inoculum on the residual amount of 22:6*n*-3. When the initial 22:6*n*-3 was 60 µg/mL, the residual 22:6*n*-3 was affected by the proportion of inoculum, uRF and water ($P < 0.05$). When no uRF was present in the mixture (i.e. 0.45 of water the incubation media) the residual amount of 22:6*n*-3 was 55.4 µg/mL (Table 3).

Table 3. Model to determine the residual amount of 22:6*n*-3 (µg/mL) after 24-h incubation of mixtures of rumen inoculum, uncentrifuged-autoclaved rumen fluid (uRF) and water in proportions ranging from 0.05 to 0.50, 0.00 to 0.45 and 0.00 and 0.45 of the total incubation liquid, respectively. The initial amount of 22:6*n*-3 was 60 and 100 µg/mL (n=3). (experiment 2)

	Initial 22:6*n*-3 concentration (µg/mL)					
	60			100		
Coefficient	*β*[1]	SE[2]	P-value	*β*[1]	SE[2]	P-value
Inoculum	4.55	1.50	0.006	25.90	3.98	0.001
uRF	10.15	1.50	0.001	30.93	3.98	0.001
Water	55.36	3.07	0.002	109.57	8.13	0.003
uRF x water	-78.36	6.63	0.001	-90.48	35.23	0.106
Inoculum x water	-56.03	8.23	0.008	-44.05	17.62	0.022
Inoculum x uRF	-21.95	6.63	0.003	-66.30	20.19	0.024

[1] Coefficient from model.

[2] Standard error of the *β* value.

The residual amount of 22:6*n*-3 decreased to 10 µg/mL and further to 4.6 µg/mL when water was replaced with uRF and rumen inoculum, respectively. A synergistic effect was observed when water was combined with uRF or rumen inoculum and when uRF was combined with rumen inoculum as indicated by the significant interaction terms (Table 3). This means that replacing water with uRF or rumen inoculum decreased the residual 22:6*n*-3 to a larger extent than would be expected just by averaging the disappearances of the single components of the model.

When the initial 22:6*n*-3 was 100 µg/mL, the residual amount of 22:6*n*-3 was greater as compared to when the initial 22:6*n*-3 concentration was 60 µg/mL (Table 3). However, the patterns of the changes in residual 22:6*n*-3 due to changes in rumen inoculum, uRF or water were similar irrespective of the initial 22:6*n*-3 concentration. When only water was present in the mixture (i.e. 0.45 (v/v) of the incubation media), residual 22:6*n*-3 was 109.6 µg/mL (Table 3). The residual amount of 22:6*n*-3 decreased to 30.9 µg/mL and further to 25.9 µg/mL when water was replaced with uRF and rumen inoculum, respectively. A synergistic effect was observed when water was replaced with inoculum ($P = 0.022$) and uRF was replaced with inoculum ($P = 0.024$) as indicated by the significant interaction term. Mixture contour plots, depicting these results, are provided as Supplementary Material (Figure S2).

Dilution-to-enrichment experiment with ruminal inoculum

22:6n-3 metabolism

Residual 22:6*n*-3 after 48-h of incubation along with the total VFA production is shown in Figure 1. The amount of 22:6*n*-3 progressively accumulated as the inoculum dilution increased. About 80% of 22:6*n*-3 was metabolized at the dilution level of 10^2, and this was reduced to 40% and 30% at the dilution level of 10^4 and 10^6, respectively. High variation among the replicates was observed at the dilution level 10^8. Only in one replicate of this dilution level, disappearance of 22:6*n*-3 was observed whereas the two other replicates failed to show disappearance of 22:6*n*-3. Disappearance of 22:6*n*-3 was not observed at the dilution levels of 10^{10}, 10^{12} and 10^{14}.

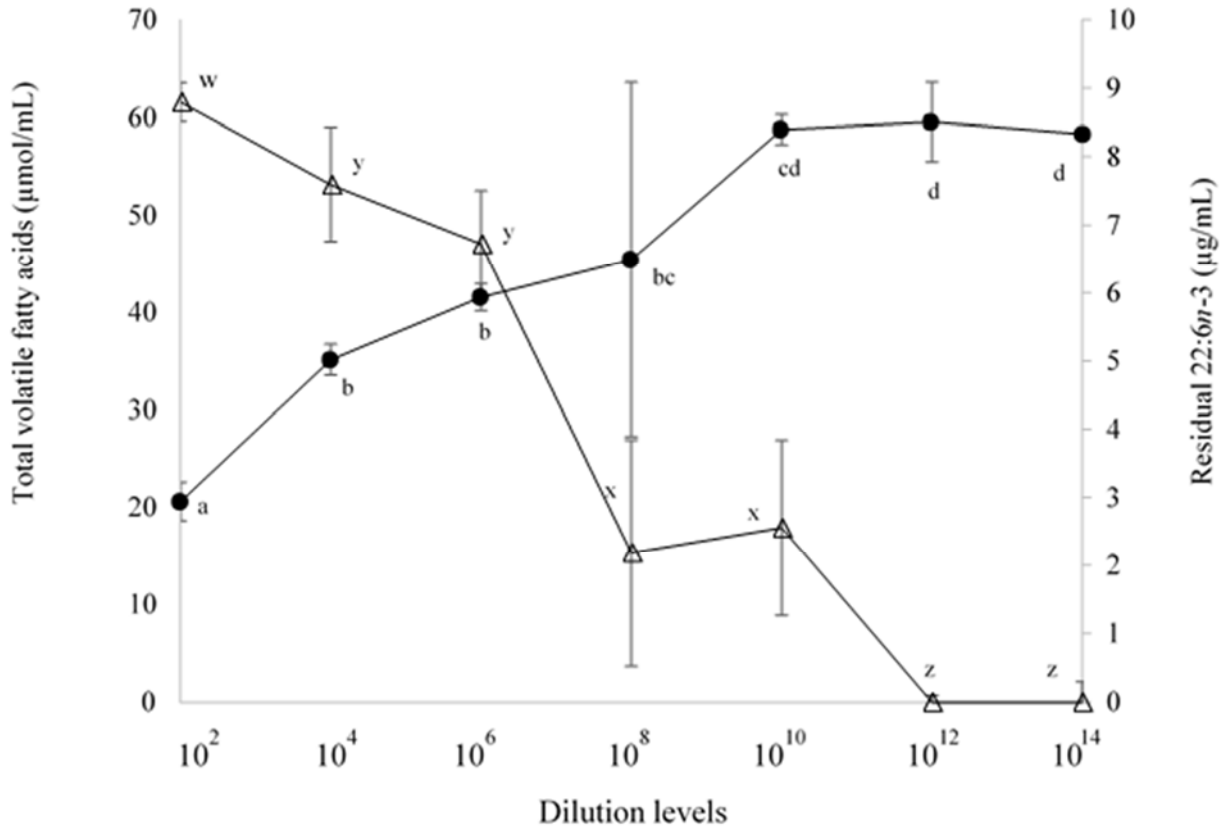

Figure 1. Metabolism of 22:6*n*-3 and VFA production at different dilutions of rumen inoculum after 48-h of incubation (experiment 3). Autoclaved-uncentrifuged rumen fluid (uRF) represented half of the incubation medium. Hydrogen was used as the headspace gas. ●Residual 22:6*n*-3 in the tube and ∆ VFA produced. Results are means and SD from 3 replicates. Within the parameter 'residual 22:6*n*-3' or 'accumulated VFA', data points with different letters are significantly different ($P < 0.05$).

When the incubation duration was increased to 144-h, the disappearance of 22:6*n*-3 increased at the dilution level of 10^2, 10^4, 10^6 and 10^8 as compared to the disappearance at 24 and 48-h (Table S2). In the 144-h incubation, high variation among replicates was also observed at the dilution level of 10^8 (Table S2). The total VFA production followed the same pattern as 22:6*n*-3 disappearance (Figure 1) with

high variation among replicates at the dilution level of 10^8. At the dilution level of 10^{10}, VFA production was still observed whereas no 22:6*n*-3 metabolism was observed. Total VFA production also increased with the incubation duration, except for the 10^{12} and 10^{14} dilutions (Table S2). We selected the samples from 48-h of incubation for further analysis of the microbial composition.

Total bacterial community structure

DGGE

We generated DGGE fingerprints of partial 16S rRNA gene fragments from the mixed bacterial communities in selected samples of dilution tubes (Figure 2A). As we expected, the number of bands decreased with increasing dilution. Bands B1-B4 and B6 were observed in all the samples of 10^2, 10^4 and 10^6 dilutions, dilutions in which disappearance of 22:6*n*-3 was observed. Some of these bands disappeared at the dilution level of 10^8, but the effect was not consistent in all replicates. It must be noted that high variation in 22:6*n*-3 disappearance and VFA production also was observed at this level of dilution. Band B5 was more intense in one replicate at 10^8 dilution and band B7 was observed only in samples which showed 22:6*n*-3 disappearance. In addition, intensity of some bands increased at the dilution of 10^{10}, and at this level VFA production was observed without 22:6*n*-3 disappearance.

Cluster analysis (Figure 2B) showed most of the samples clustered by dilution, which largely coincides with the residual 22:6*n*-3. For dilution 10^8, when residual 22:6*n*-3 largely varied across replicates (Figure 1), clustering is particularly linked with the residual 22:6*n*-3. At the dilution level of 10^8, two of the replicates (lanes 9 and 10) which had higher residual 22:6*n*-3 (8.2 and 7.7 µg/mL) clustered together (82%

similarity) away from the other replicate (lane 8) which had lower residual 22:6*n*-3 (3.5 µg/mL). The microbial profile from lane 8 shared some bands with previous dilutions (B1, B2, B4 and B7). The intensity of these bands was reduced in lane 10 and totally disappeared in lane 9 and in further dilutions. Bands present at 10^{10} dilution, when no disappearance of 22:6*n*-3 was observed, are unlikely to be related to 22:6*n*-3 metabolism.

To identify potential 22:6*n*-3 biohydrogenating bacteria, some of the bands which were not present anymore at the dilution level where 22:6*n*-3 disappearance was no longer observed were excised from DGGE gels (B1-B7 in Figure 2A) and sequenced (Table 4). A phylogenetic dendrogram was constructed to display the apparent relatedness of the sequences to each other and to reference sequences (Figure 3). Bands 1 and 2 were associated to the genera *Streptococcus,* while bands 6 and 7 are closely related to genera *Selenomonas ruminantium.* Band 4 is related to *B. proteoclasticus*. Bands 3 and 5 are associated to uncultured bacteria.

Table 4. Closest relatives of bacterial species or bacterial clone represented in excised DGGE bands. GenBank accession numbers are given in parenthesis.

Band Number	Closest bacterial species or clones
B1	100% *Streptococcus gallolyticus* (CP013688)
B2	93% Streptococcus sp. (AB371971)
B3	92% uncultured Bacteroidetes bacterium RM23 (AB730682)
B4	95% Uncultured Butyrivibrio sp. (AB849434)
B5	90% Uncultured rumen bacterium RC115 (AB615042)
B6	100% Selenomonas ruminantium Y6 (EU327402)
B7	98% Uncultured rumen bacterium (KF698307)

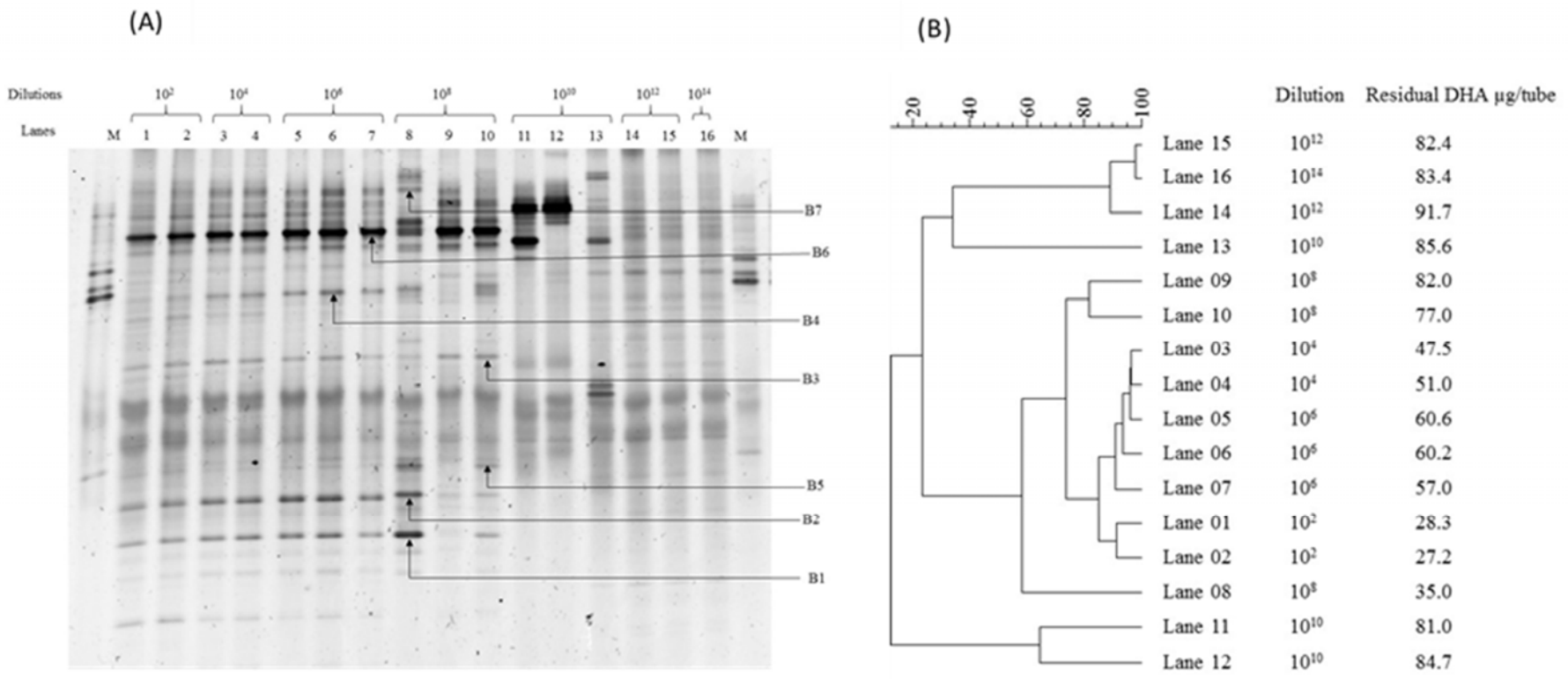

Figure 2(A). Bacterial 16S rRNA DGGE fingerprints of selected samples from dilution tubes after 48-h of incubation (experiment 3). The labelled arrows indicate the bands that were excised and sequenced. The marker (M) consisted of amplicons of 16S rRNA genes from 6 pure culture of bacteria obtained from our lab (*A. salmonicida, V. anguillarum*-VQ775, *S. agalactiae*, *V. herveyi, V. anguillarum*-NB10 and *B. proteoclasticus* P-18).

Figure 2(B): The similarities (Pearson's correlation %) of the DGGE fingerprints of figure 2A.

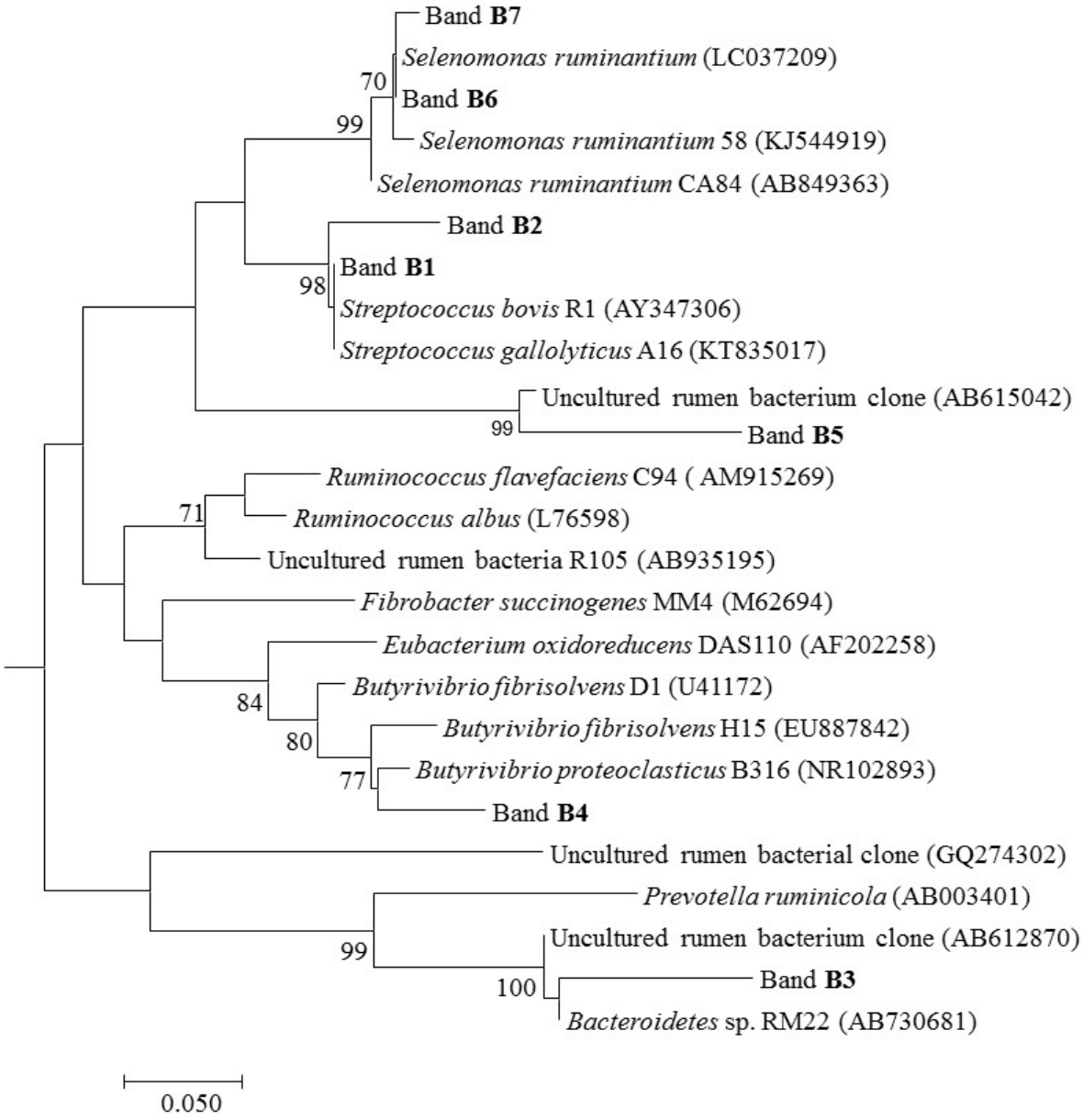

Figure 3. Inferred phylogenetic relationships between partial 16S rRNA genes derived from excised bands (bold) and reference 16S rRNA gene sequences (experiment 3). In parenthesis shown the GenBank accession numbers. The tree was constructed from an alignment of 180 nucleotide positions. Bootstrap values (>70%) are shown at selected nodes and are percentages based on 1000 resampling (Felsenstein, 1985). The scale bar indicates 0.05 nucleotide substitutions per nucleotide position. The 16S rRNA gene sequences of *Methanobrevibacter gottschalkii* strain PG (U55239) and *Methanobrevibacter* sp. strain SM9 (AJ009958) were used to root the Dendrogram (not shown).

16S rRNA amplicon sequencing

All samples from the 48-h incubation period (n=21) were used for 16S rRNA amplicon sequencing. The observed richness of OTU's and Shannon-Wiener and Simpson diversity indices decreased with increasing dilution from dilution 10^2 to 10^8 (Figure 4). High variation of these indices was observed at the dilution level of 10^{10}. Examination of the relative abundances showed that 10^{10} had high proportion of Fibrobacteres (35%) in only one of the tubes. Because in this replicate there was also no VFA production, we considered this replicate as an outlier and removed it from further analysis. A dendrogram of the community composition of the microbiota confirmed the observation of the DGGE pattern (Figure 5). This dendrogram was limited to dilutions 10^2 to 10^{10}. Dilutions 10^{12} and 10^{14} were excluded, as a preliminary heatmap analysis including these dilutions revealed a distinct cluster of those samples (data not shown). As no 22:6*n*-3 metabolism and - more importantly - no VFA production was observed at these dilution levels, complete absence of bacterial growth is suggested in these dilutions which implies the bacterial DNA likely comes from the bacterial particles of the uRF used for the media preparation (50% v/v). For the same reason, these dilutions have not been presented in Figure 4.

Samples clustered by dilution level except for the dilutions 10^8 and 10^{10}. Sample clustering was linked to the residual 22:6*n*-3 in dilution level 10^8. Like in the DGGE fingerprinting clustering, two of the replicates (Dil 8-2 and Dil 8-3, Figure 5) which had higher residual amounts of 22:6*n*-3 clustered together, away from the third replicate (Dil 8-1) which had a lower residual amount of 22:6*n*-3. Clustering of the samples from dilution level 10^{10} also follows the same pattern as in DGGE fingerprint clustering.

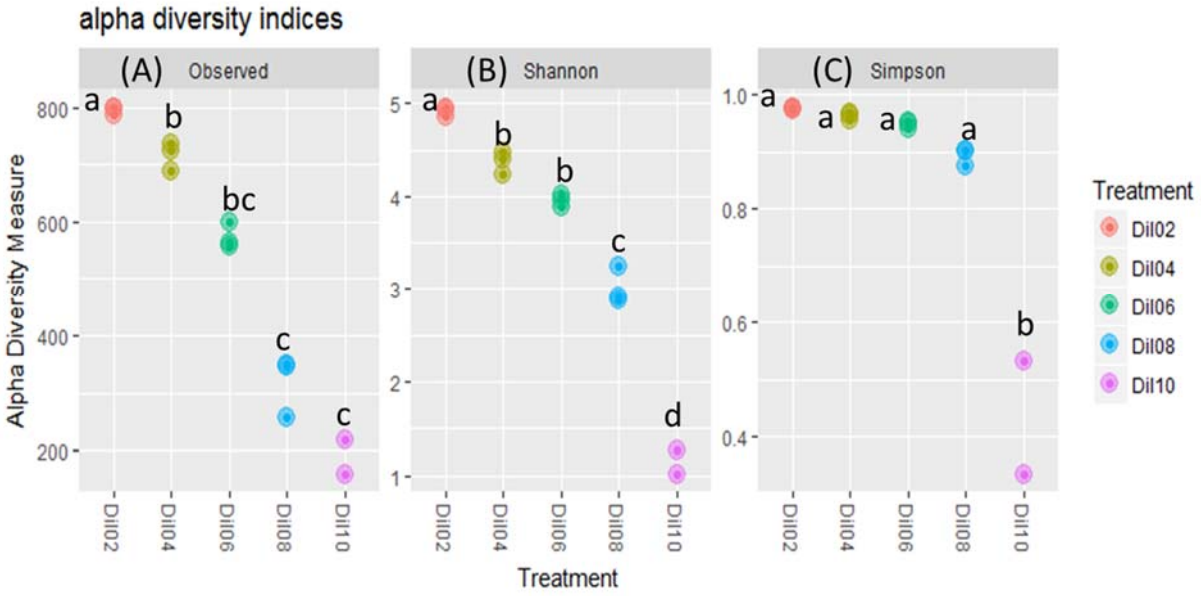

Figure 4. Graphical representation of the (A) Richness (number of observed OTUs), Shannon diversity (B) and Simpson diversity (C) indices of the bacterial communities at different dilution levels (10^2 to 10^{10}, indicated here as Dil2 to Dil10) in the dilution-to-enrichment after 48-h of incubation (experiment 3). The letters (a, b, c) indicate the statistical classification for each dilution level. Samples without common letters are significantly different ($P < 0.05$).

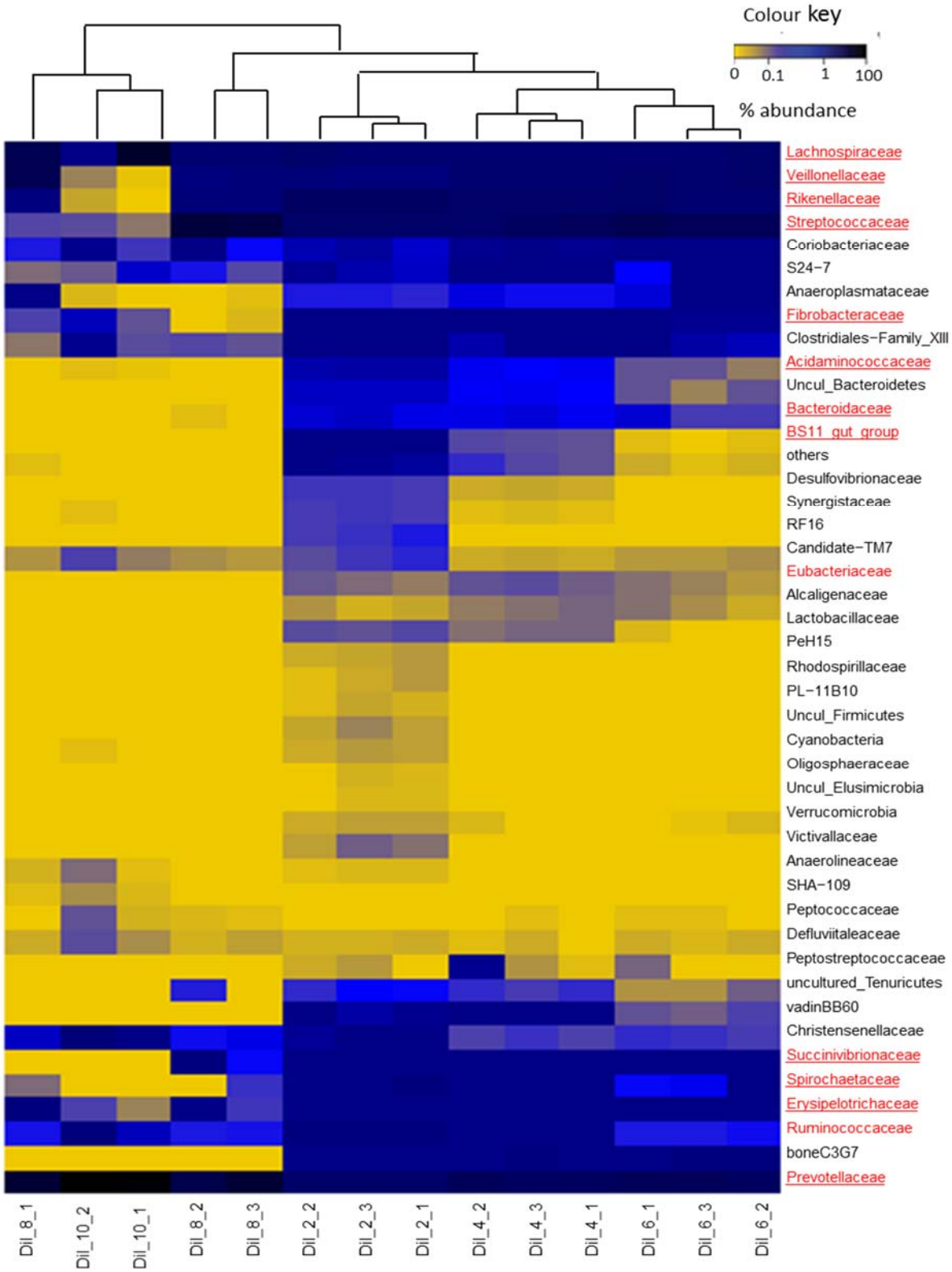

Figure 5. Heatmap of the bacterial composition at family level of samples from dilution tubes ((10^2 to 10^{10}, indicated here as Dil_2 to Dil_10) after 48-h of incubation experiment 3). Each dilution contained three replicates indicated by numbers 1 to 3 associated to the dilution coding. The dendrogram indicates the community resemblance between samples based on UPGMA clustering and Manhattan distance methods. The colour code indicates de relative abundance (log scale). In red letters indicated bacteria at family level referred n Table 5.

The average relative abundances of each phylum and the major families as well as some genera are summarized in Table 5. Phylum *Firmicutes* was the most abundant phyla in the dilution levels 10^2-10^8 (≈50%), but its contribution reduced to 30% at the dilution level of 10^{10}. Within this plylum, the *Lachnospiracea* family was not correlated with the residual 22:6*n*-3, but a closer look into this family showed that the abundance of the genus *Pseudobutyrivibrio* was negatively correlated with the residual 22:6*n*-3 (R=-0.73, P < 0.01). The relative abundance of *Butyrivibrio* genus was not correlated with residual amount of 22:6*n*-3. The family *Veillonellaceae* was highly present in the dilution levels of 10^2 to 10^8 with *Selenomonas* being the major genus here. Nevertheless, there was no significant correlation between *Selenomonas* with the residual 22:6*n*-3 (Table 5).

Abundance of the genera *Acidaminococcaceae* (R = -0.74, P < 0.01) and *Erysipelotrichaceae* (R = -0.81, P < 0.01) was also negatively correlated with the residual 22:6*n*-3. In contrast, abundance of the genus *Streptococcus* (family *Streptococcaceae*) did not show a significant correlation with the disappearance of 22:6*n*-3, although it was present in relatively high numbers in the 10^2 to 10^8 dilutions and reduced in the 10^{10} dilution.

The second dominant phylum in the dilution-enrichment samples was *Bacteroides* (≈40%), and *Rikenellaceae and Prevotellaceae* were the *most* abundant families within this phylum. *Rikenellaceae* negatively correlated with residual 22:6*n*-3. A closer look showed that the uncultured RC9-gut group (R= -0.77, P < 0.01) was mainly responsible for this negative correlation.

Table 5. Overview of the average relative abundance (%) of the bacterial phyla and major families in the different levels of dilution-enrichment cultures supplemented with 22:6*n*-3. Additionally, within families, some of the more predominant genera, genera known as potential biohydrogenators or genera showing significant correlations with the residual 22:6*n*-3 are reported. Spearman's rank correlation between the relative abundance of each taxa and the residual amount of 22:6*n*-3 in the different cultures at various dilution levels (10^2 to 10^{10}) (n = 3 per dilution level).

	Average relative abundance (%) Dilution level					Spearman	
Taxonomy	10^2	10^4	10^6	10^8	10^{10}	R	P
Firmicutes	46.25^{a}	49.62^{a}	54.47^{a}	56.05^{a}	30.0^{b}	-0.51	0.02
Streptococcaceae	14.10^{ab}	17.42^{ab}	22.80^{a}	21.97^{a}	1.14^{b}	-0.34	NS
Streptococcus	13.91^{bc}	17.42^{bc}	22.80^{ab}	21.09^{ab}	1.09^{c}	0.27	NS
Lachnospiracea	12	12	13.12	16.39	20.81	0.01	NS
Pseudobutyrivibrio	4.27^{ab}	3.98^{ab}	3.65^{ab}	6.41^{a}	0^{b}	-0.73	<0.01
Butyrivibrio	0.77	0.47	0.29	1.27	19.95	-0.25	NS
Veillonellaceae	6.60^{ab}	11.23^{a}	13.66^{a}	13.83^{a}	0^{b}	-0.13	NS
Selenomonas	5.79^{abc}	10.35^{b}	13.24^{a}	13.29^{a}	0^{c}	-0.18	NS
Ruminococcaceae	4.93^{a}	1.43^{bc}	0.46^{c}	0.45^{c}	3.09^{ab}	-0.42	NS
Acidaminococcaceae	0.91^{a}	0.59^{b}	0.12^{c}	0^{d}	0.01^{d}	-0.74	<0.01
Erysipelotrichaceae	3.44^{a}	1.94^{ab}	2.63^{ab}	2.59^{ab}	0^{b}	-0.81	<0.01
Eubacteriaceae	0.08^{ab}	0.12^{a}	0.06^{b}	0^{c}	0^{c}	-0.64	<0.01

Table 5. (Continued)

Taxonomy	Average relative abundance (%) Dilution level 10^2	10^4	10^6	10^8	10^{10}	Spearman R	Spearman P
Bacteroidetes	37.40[a]	38.08[a]	33.98[a]	40.6[a]	68.11[b]	-0.05	NS
Rikenellaceae	18.32[a]	16.20[ab]	13.02[b]	7.64[c]	0.03[d]	-0.79	<0.01
RC9-gut group	17.3[a]	15.83[ab]	12.97[b]	7.64[c]	0[d]	-0.77	<0.01
Prevotellaceae	14.55[a]	19.24[a]	19.43[a]	32.71[b]	67.63[c]	0.76	<0.01
BS11 gut group	1.72[a]	0.17[b]	0.01[b]	0[b]	0.02[b]	-0.77	<0.01
Bacteroidaceae	0.72[a]	0.65[ab]	0.41[b]	0.01[c]	0[c]	-0.62	<0.01
Spirochaetae	4.05[a]	2.56[b]	0.87[cd]	0.13[d]	0[d]	-0.86	<0.01
Spirochaetaceae	4.03[a]	2.56[b]	0.87[cd]	0.13[d]	0[d]	-0.86	<0.01
Proteobacteria	3.92[a]	2.05[ab]	2.13[ab]	1.58[b]	0.35[b]	-0.42	0.12
Succinivibrionaceae	3.59[a]	1.91[ab]	2.07[ab]	1.58[ab]	0[b]	-0.38	0.17
Fibrobacteres							
Fibrobacteraceae	2.69[a]	1.61[b]	1.08[bc]	0.08[cd]	0.34[c]	-0.76	<0.01

Superscript letters indicate the statistical classification in homogeneous abundance groups. Dilution levels without common superscript are significantly different (P≤0.05).

Two additional phyla, *Spirochaetae* and *Proteobacteria,* contributed only about 4% of the total bacteria in the samples of dilution level 10^2. Their contribution reduced with the dilution level until the dilution level of 10^{10}. *Spirochaetaceae* and *Succinivibrionaceae* were the major families of these phyla and only *spirochaetaceae* was negatively correlated with residual 22:6*n*-3.

Pure culture experiment

Close relatives of the bacterial species cloned from the DGGE bands, which were available in the public culture collection (DSMZ) were purchased and cultivated according to the DSMZ specified culture conditions. Two *Selenomonas ruminantium* strains (*S. ruminatium lactilytica* DSM 2872 and *S. ruminantium ruminantium* DSM 2150) were available for this purpose (B6 and B7 in Figure 2A) and *Streptococcus gallolyticus* (B1 in Figure 2A). After cultivation, the ability of these strains to metabolize 22:6*n*-3 was assessed.

Neither *Selenomonas* strains (*S. ruminatium lactilytica* DSM 2872 and *S. ruminantium ruminantium* DSM 2150) nor *Streptococcus gallolyticus* metabolized 22:6*n*-3 at a concentration of 5, 10 and 20 µg/mL. However, their VFA production or their growth were not affected by any of the 22:6*n*-3 concentrations (data not shown). Unfortunately, bands B3, B5 and B7 were most closely related to uncultured rumen bacteria which impeded their assessment for 22:6*n*-3 biohydrogenation.

DISCUSSION

Effect of uRF on the disappearance of 22:6*n*-3

Biohydrogenation of 22:6*n*-3, as evaluated by the residual amount of 22:6*n*-3, was affected by the addition of uncentrifuged-autoclaved rumen fluid (uRF). When uRF was not present, reducing the inoculum size decreased the disappearance of 22:6*n*-3 in agreement with previous studies (Vlaeminck *et al*., 2014). In contrast, when uRF was added, reducing the amount of rumen inoculum was not accompanied with a decrease in the disappearance of 22:6n-3. These preliminary results of experiment 1 provided the first indication of a significant effect of uRF to stimulate disappearance of 22:6*n*-3. The robustness of this effect was further assessed in a second experiment which included mixtures of different proportions of uRF, inoculum, water and buffer, and two different 22:6*n*-3 concentrations. Results of this more comprehensive experiment confirmed uRF exerts a robust effect on increasing the disappearance of 22:6*n*-3.

The increased disappearance of 22:6*n*-3 upon addition of uRF might be due to a reduction of the 'effective' concentration of 22:6*n*-3 in the incubation media. Indeed, residual feed particles present in uRF potentially provide an important site for adsorption of fatty acids (Harfoot *et al*, 1973) resulting in a reduced amount of 22:6*n*-3 to effectively interact with the bacteria. In the study of Chapter 1 of this thesis, addition of adsorbents stimulated the disappearance of 22:6*n*-3 in incubations with rumen inoculum. However, the adsorbents in the latter study also enhanced VFA production as the adsorbents apparently also provided fermentable substrate, which was reflected in a positive relationship between 22:6*n*-3 biohydrogenation and VFA

production. In the current experiment, the role of uRF as a supplier of fermentable substrate cannot be excluded as a positive correlation between VFA production and 22:6*n*-3 biohydrogenation was also observed here (Figure S3).

The vast stimulating effect of uRF on the disappearance of 22:6*n*-3 even at low levels of rumen inoculum is of particular interest within the context of the dilution-to-enrichment experiment in which reduced inoculum sizes are used.

Identification of the bacterial species involved in the biohydrogenation of 22:6*n*-3.

Molecular techniques such as DGGE and next generation sequencing (NGS) were used by several researchers to study changes in the rumen microbial community upon supplementation of 22:6*n*-3 (Boeckaert *et al*., 2008; Huws *et al*., 2011; Kim *et al*., 2008). Nevertheless, these studies investigated the effect of 22:6*n*-3 supplementation on the biohydrogenation process of other PUFA (i.e. inhibition of 18:0 formation) and not the bacteria involved in 22:6*n*-3 biohydrogenation.

In order to identify bacterial species able to biohydrogenate 22:6*n*-3, we applied the dilution-to-enrichment technique using growth media containing uRF. We hypothesized that the bacterial species able to hydrogenate or resistant to 22:6*n*-3 will dominate the cultures, especially at higher inoculum dilutions. PUFA such as 22:6*n*-3, are known to be toxic to rumen bacteria and biohydrogenation is suggested to be a detoxification mechanism by rumen bacteria. To detect the bacterial composition at each inoculum dilution level, we used DGGE and 16S rRNA amplicon sequencing. Clustering of samples based on the bacterial composition (DGGE and 16S rRNA amplicon sequencing) showed that at the first inoculum-dilution steps (10^2,

10^4 and 10^6), all the replicates from each dilution level clustered together. When the inoculum was highly diluted (dilution levels 10^8 and 10^{10}), the bacterial composition differed among replicates which was accompanied with high variation in VFA production and residual 22:6*n*-3.

PCR-DGGE bands exhibiting changes along the serial dilution accompanied by a decrease of 22:6*n*-3 metabolism were of interest as potential biohydrogenating 22:6*n*-3 candidates. The most interesting changes in DGGE profiles were at dilution 10^8 in which the disappearance of several bands was associated with variation in 22:6*n*-3 metabolism. Three bands were obtained from the bacterial community analysis which could be related to the 16S rRNA genes of known species, whereas the other bands corresponded to yet unclassified bacteria. One of the bands (B4) was identified to be member *Butyrivibrio* sp. *Butyrivibrio* species are well known to be actively involved in the biohydrogenation process of PUFA. This band was observed in all the lanes till the dilution level of 10^6, and in the dilution level of 10^8 this band only clearly appeared in lane 8 which is associated with a replicate in which 22:6*n*-3 biohydrogenation occurred. At further dilution levels, the band B4 disappeared which could suggest the involvement of this bacteria in 22:6*n*-3 biohydrogenation. Nevertheless, the relative abundance of the genus *Butyrivibrio* did not show any correlation with the residual amount of 22:6*n*-3. However, the relative abundance of the genus *Pseudobutyrivibrio* showed a significant negative correlation with the residual amount of 22:6*n*-3. The role of these genera in PUFA biohydrogenation was suggested by Boeckaert et al. (2008) when *Butyrivibrio*-like bacteria were associated with changes in the rumen community of cows fed algae resulting in *trans*-11 18:1 and *trans*-10 18:1 accumulation (Boeckaert *et al*., 2008). Moreover, in our study, the group *Rikenellaceae* showed a negative correlation with the residual amount of

22:6*n*-3. *Rikenellaceae* has not been extensively studied in the rumen, but Zened et al. (2013) already reported PUFA supplementation to affect the relative abundance of *Rikenellaceae* (as well as *Butyrivibrio-Pseudobutyrivibrio*): the abundance of these bacterial groups were much lower in rumen contents of cows receiving sunflower oil than their controls (Zened *et al*., 2013).

The most intense band (B6) observed in low dilution inoculum samples (up to 10^8 dilutions) was *S. ruminantium* and this species disappeared when 22:6*n*-3 was no longer metabolized (from dilution 10^{10} onwards). This was confirmed by NGS data, although the relative abundance of the genus *Selenomonas* (family *Veillonellaceae)* was not correlated with the residual amount of 22:6*n*-3. Both the DGGE pattern as well as the NGS relative abundance data indicate an enrichment of this genus at increasing dilutions, which could suggest *Selenomonas* to be quite resistant to 22:6*n*-3 toxicity. Some bacterial species are naturally resistant to the antibacterial action of FFA and they can grow in the presence of PUFA (Desbois and Smith, 2010). Differential susceptibility of bacterial species to the action of FFA is likely to be due to the FFA's ability to permeate the outer membrane or cell wall, which will enable access to the sites of action on the inner membrane. For example, resistant bacteria to the presence of 22:6*n*-3 are *S. ruminantium*, *S. bovis*, *A. lipolytica* (Maia et al., 2007). Indeed, observations by Maia et al. (2007), showed that the growth of *Selenomonas ruminantium* was not inhibited when incubated with 50 µg/mL of 22:6*n*-3. This bacterium generally seemed insensitive to several PUFA. *S. ruminantium* is a non-fibrolytic bacteria that ferment soluble carbohydrates in the rumen (Tajima *et al*., 2001) and was reported to be a predominant bacterium in continuous cultures when incubated with fish oil (Potu *et al*., 2011). Based on pure culture studies, Hudson et al. (1995) reported hydration activity of *S. ruminantium* which converted oleic acid

into 10-hydroxystearic acid. However, this rumen bacterium did not show biohydrogenation activity (Hudson *et al*., 1995). In our pure culture study, *S. ruminantium* did not metabolize 22:6*n*-3 suggesting that it is not involved in the disappearance of 22:6*n*-3. Hence, the increase of *Selenomonas* with increasing dilution was probably due to their resistance to PUFA toxicity rather than their capacity to hydrogenate.

Bands 1 and 2 were related to *Streptococcus* sp. with band 1 being identified as *S. gallolyticus*. *S. gallolyticus* (formally known as *Streptococcus bovis* biotype 1) is a common rumen inhabitant. Recent genome analysis revealed that *S. gallolyticus* has a versatile lifestyle, with the capacity to adapt to different environments and it was also linked to detoxification processes (Rusniok *et al*., 2010). In terms of FA metabolism, it is known as a hydrating bacterium and one study showed the ability to hydrogenate vaccenic acid (*trans*-11 18:1; VA). *S. bovis*, also has the capacity to hydrate 18:2*n*-6 to 13-hydroxy-9-octadecenoic acid, thus diverting it away from the biohydrogenation pathway (Hudson *et al*., 1998). However, the contribution of *S. bovis* to the metabolism of unsaturated fatty acids in the rumen is probably limited as its population size in omasal digesta was rather small (0.01% of total bacteria), with no obvious relation with the flow of hydroxyacids at the omasum. As for the *Selenomonas* genus, the enrichment of *Streptococcus* at higher dilution levels might be related to its resistance to 22:6*n*-3 toxicity as Maia et al. (2007) also showed that the growth of S. *bovis* was not inhibited when incubated with 50 µg/mL of 22:6*n-3*.

Accordingly, the dilution technique rather might have resulted in enrichment of bacteria which are resistant to 22:6*n*-3 than of bacteria which are able to biohydrogenate 22:6*n*-3. As biohydrogenation does not represent a metabolic

advantage, because bacteria do not generate energy from biohydrogenation, it is possible that bacteria able to biohydrogenate were outcompeted by other organisms which are more resistant to 22:6*n*-3.

SUPPLEMENTARY INFORMATION CHAPTER 2

Table S1. Primers used in experiment 3 for amplification of 16S rRNA of total Bacteria for DGGE and amplicon sequencing

Target gene	Primers	Purpose	Primers Sequence (5′-3′)	Reference
16S rRNA bacteria	338F-GC*	DGGE	ACTCCTACGGGAGGCAGCAG	Muyzer et al., 1993
	518R		ATTACCGCGGCTGCTGG	
	344F	NGS	CCTACGGGNGGCWGCAG	Klindworth et al., 2013
	806r		GACTACHVGGGTATCTAATCC	

The forward primer (GC-338F) included an additional 40-bp GC rich sequence segment (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCA CGG GGG G) at the 5´ end.

Table S2. Residual 22:6*n*-3 and total volatile fatty acid production after 24-h, 48-h and 144-h of incubation with initial supplementation of 22:6*n*-3 (10 µg/mL) in enrichment cultures incubated with diluted rumen fluid inoculum, with dilution levels ranging from 10^2 to 10^{14}.

	Dilution levels						
	10^2	10^4	10^6	10^8	10^{10}	10^{12}	10^{14}
Residual 22:6*n*-3 (µg/mL)							
24-h	4.37 ± 0.05^{b}	6.68 ± 0.14^{ab}	7.12 ± 0.18^{a}	7.44 ± 0.51^{a}	8.06 ± 0.09^{a}	8.58 ± 0.07^{a}	8.54 ± 0.16^{a}
48-h	2.94 ± 0.29^{b}	5.01 ± 0.23^{c}	5.93 ± 0.20^{c}	6.49± 2.60^{d}	8.4 ± 0.23^{ad}	8.51 ± 0.58^{a}	8.33 ± 0.06^{a}
144- h	0.12 ± 0.03^{b**}	2.45 ± 0.13^{c**}	3.91± 0.13^{c**}	4.15± 2.15^{c**}	8.15 ± 0.39^{a}	8.46 ± 0.17^{a}	8.50± 0.18^{a}
Total VFA production (µmol/mL)							
24-h	44.6 ± 2.0^{b}	34.9 ± 0.9^{c}	25.3 ± 5.5^{d}	4.70 ± 4.70^{a}	<0.1^{a}	<0.1^{a}	<0.1^{a}
48-h	61.6 ± 2.0^{b*}	53.1 ± 5.9^{c*}	47.0 ± 5.4^{c*}	15.3 ± 11.6^{d*}	17.9 ± 9.0^{d*}	<0.1^{a}	<0.1^{a}
144-h	72.5 ± 0.4^{b*}	69.6 ± 4.4**	61.6 ± 2.1^{c**}	45.3 ± 1.8^{d**}	27.6 ± 6.0^{e**}	<0.1^{a}	<0.1^{a}

Along the raw, data points with different letters are significantly different ($P < 0.05$). * significantly different from the 24-h data at the same dilution level. ** significantly different from 24-h and 48-h data at the same dilution level.

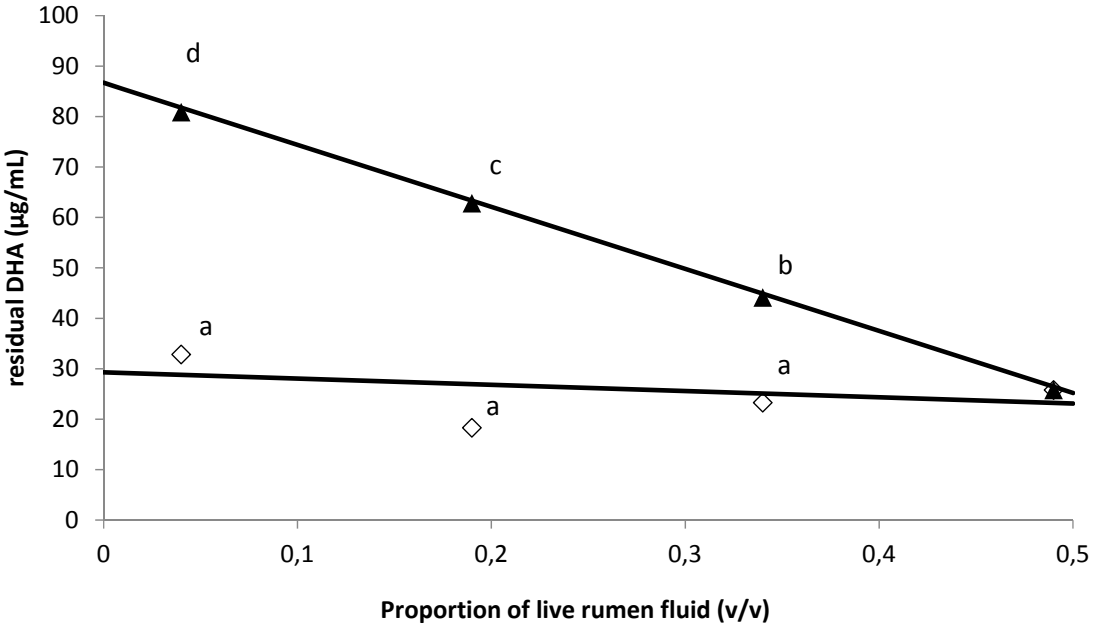

Figure S1. The effect of inoculum size and presence (▲) or absence (◊) of uncentrifuged autoclaved rumen fluid (uRF) on the residual amount of 22:6*n*-3 after a 24-h incubation period (experiment 1). The initial 22:6*n*-3 concentration was 100 µg/mL. The amount of uRF added was 0.45, 0.30 and 0.15 (v/v) to the tubes containing 0.05, 0.20 and 0.35 (v/v) inoculum, respectively. Decreasing amounts of inoculum when no uRF was present decreased the disappearance of 22:6*n*-3. When uRF was added, no difference in the residual 22:6*n*-3 was found among inoculum sizes. Each point represents the mean of 3 replicates. *Data points of inoculum with uRF differ significantly from the inoculum without uRF ($P < 0.05$).

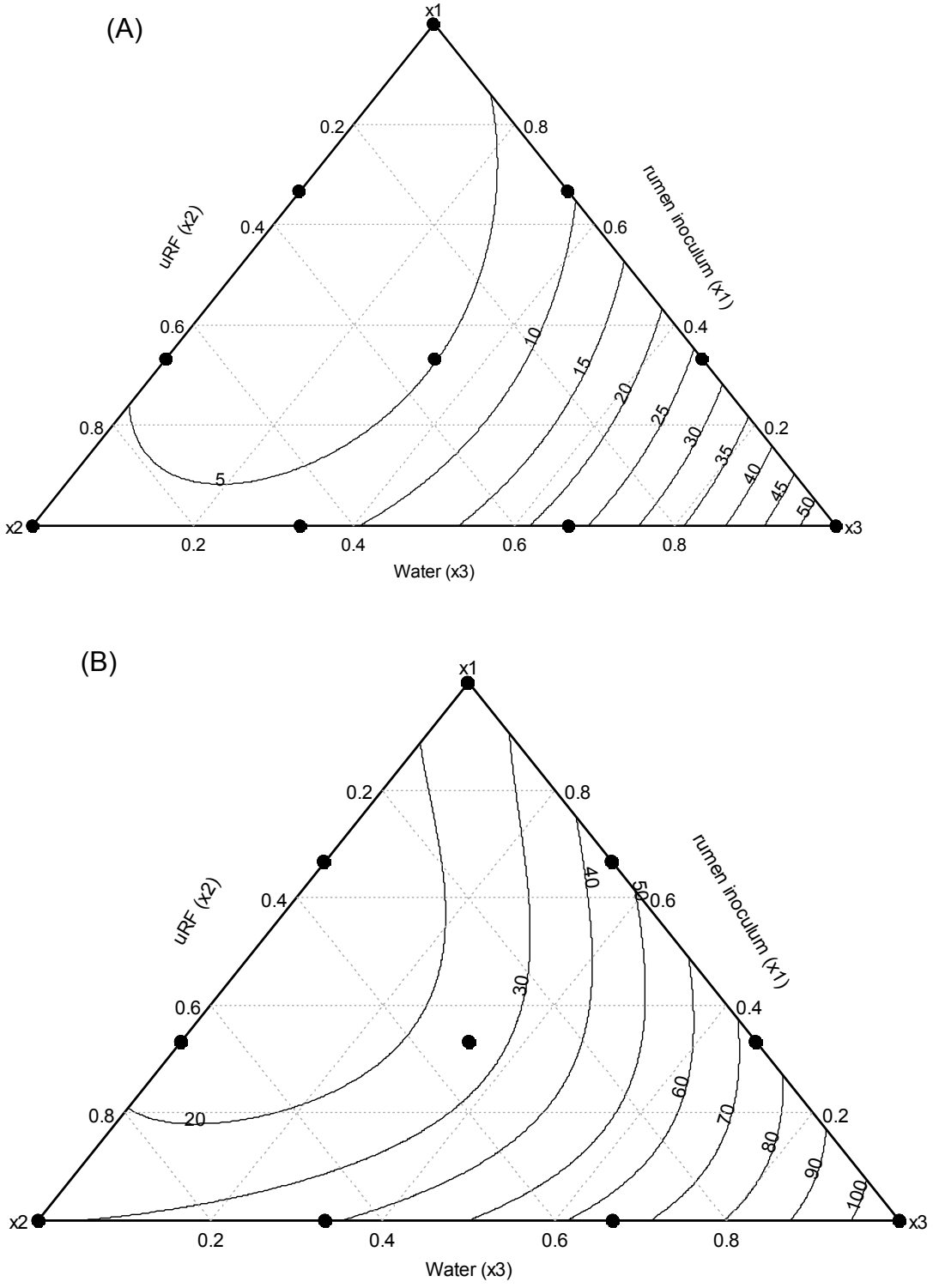

Figure S2. Mixture contour plot depicting the relation of the proportion of rumen inoculum (X1), uncentrifuged autoclaved rumen fluid (uRF, X2) and water in the mixture and residual 22:6*n*-3 (µg/mL) after 24-h of in vitro incubation (experiment 2). The initial 22:6*n*-3 concentration was (A) 60 µg/mL and (B) 100 µg/mL culture content. Coded values of rumen inoculum, uncentrifuged autoclaved rumen fluid and water were used to fit the model (see Table 1)

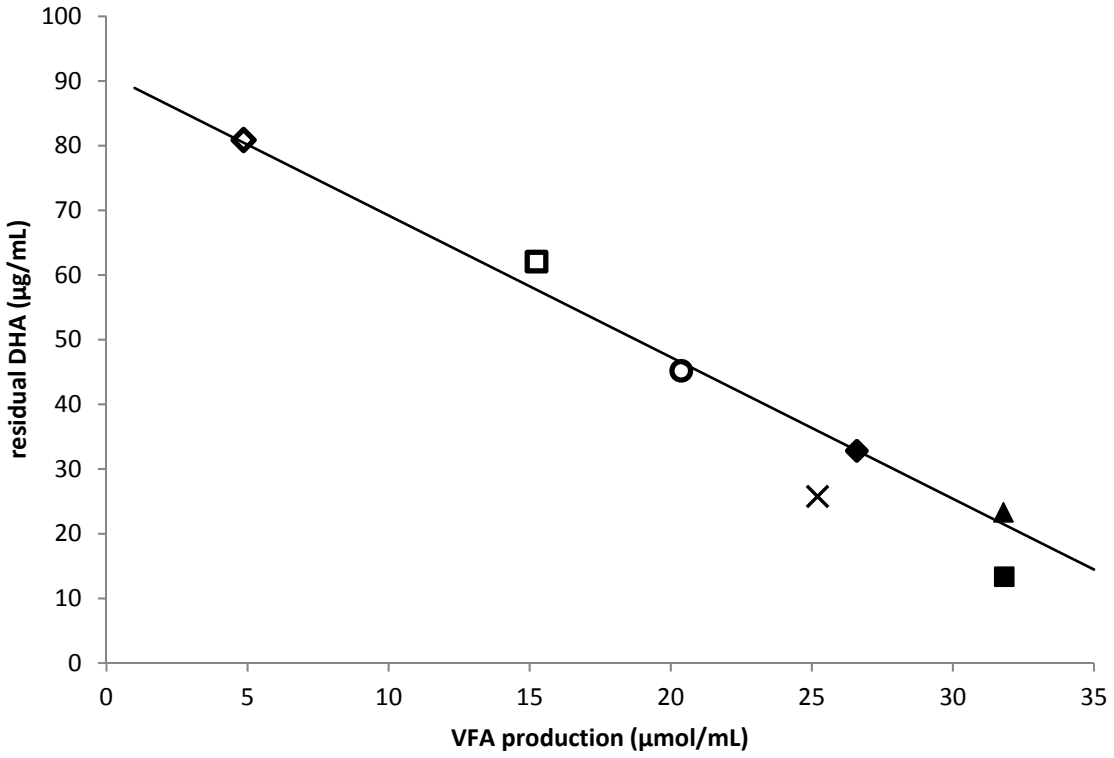

Figure S3. Association of apparent production of volatile fatty acids (VFA) and the residual amount of 22:6*n*-3 left in the incubation flask after 24-h incubation with different amounts of rumen fluid inoculum with (filled figures) or without (open figures) uncentrifuged-autoclaved rumen fluid (uRF) (experiment 1). The amounts of rumen inoculum was 0.05 (◊), 0.20 (□) and 0.35 (○) (v/v) inoculum, respectively. The initial 22:6*n*-3 concentration was 100 µg/mL. Values represent mean values (n=3). Y = 91.11 (SE=4.37, P < 0.001) – 2.19 (SE = 0.17, P < 0.001) × X.

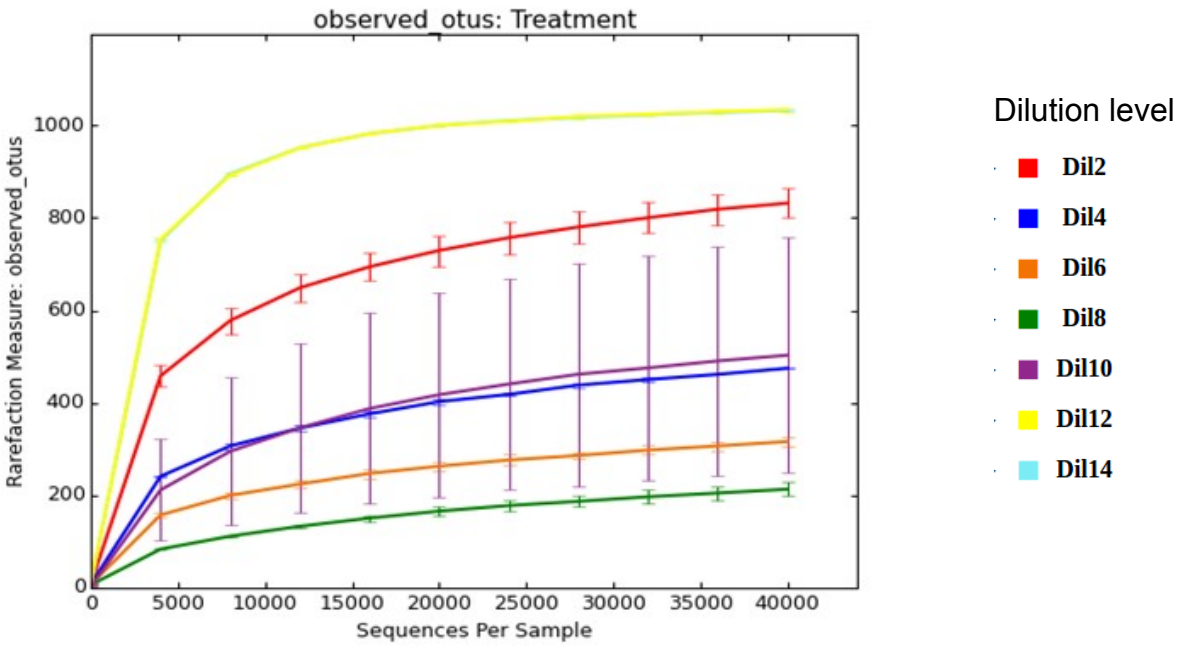

Figure S4. Rarefaction curves of alpha diversity in different dilutions (experiment 3). Number of OTU's sequenced per sample (X axis) in function of the number of OTU' observed (Y axis).

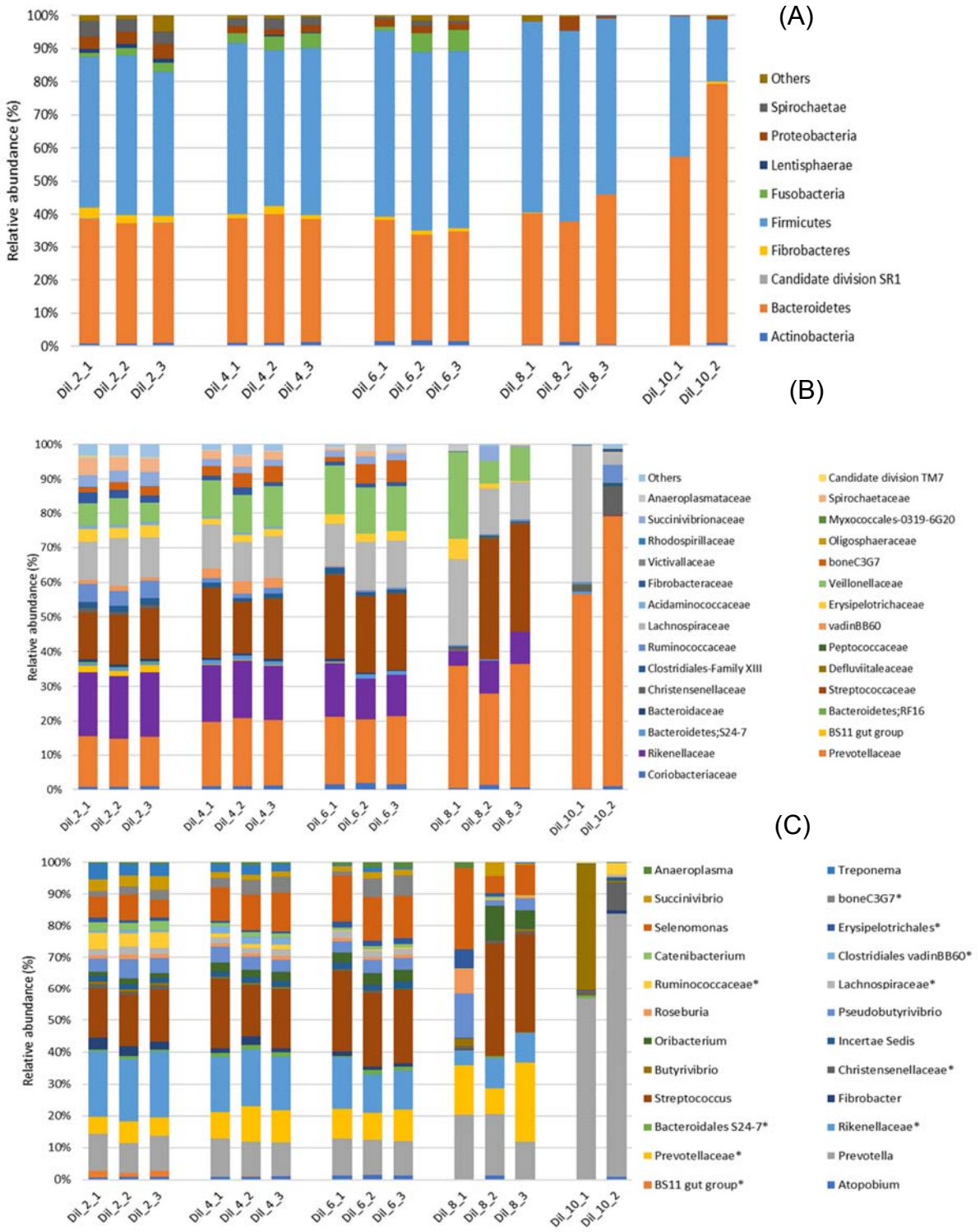

Figure S5. The relative abundance of bacterial composition obtained by 16s amplicon sequencing in diluted cultures after 48-h incubation (experiment 3) at phylum (A), family (B) and genus (C) levels.

Chapter 3: biohydrogenation of 22:6*n*-3 by *Butyrivibrio proteoclasticus* P18

Redrafted after Jeyanathan, J., Escobar, M., Wallace, R.J., Fievez, V., and Vlaeminck, B. (2016). Biohydrogenation of 22:6n-3 by Butyrivibrio proteoclasticus P18. BMC Microbiol. *16*, 104-116.

ABSTRACT

Rumen microbes metabolize 22:6*n*-3. However, pathways of 22:6*n*-3 biohydrogenation and ruminal microbes involved in this process are not known. In this study, we examine the ability of the well-known rumen biohydrogenating bacteria, *Butyrivibrio fibrisolvens* D1 and *Butyrivibrio proteoclasticus* P18, to hydrogenate 22:6*n*-3. *B. fibrisolvens* D1 failed to hydrogenate 22:6*n*-3 in growth medium containing autoclaved ruminal fluid that either had or had not been centrifuged. *B. proteoclasticus* P18 hydrogenated 22:6*n*-3 in growth medium containing autoclaved ruminal fluid that either had or had not been centrifuged. The amount of 22:6*n*-3 hydrogenated was quantitatively recovered in several intermediate products eluting on the gas chromatogram between 22:6*n*-3 and 22:0. For the first time, our study identified ruminal bacteria with the ability to hydrogenate 22:6*n*-3. The gradual appearance of intermediates indicates that biohydrogenation of 22:6*n*-3 by *B. proteoclasticus* P18 occurs by pathways of isomerization and hydrogenation resulting in a variety of unsaturated 22 carbon fatty acids.

INTRODUCTION

Docosahexaenoic acid (22:6*n*-3) is a polyunsaturated fatty acid (PUFA) that has been associated with physiological benefits in many species, including human and dairy cows. The amount of 22:6*n*-3 available for adsorption in the small intestine can be increased by intake of marine products (e.g. fish oil, marine algae). However, this is not straightforward in ruminants as extensive biohydrogenation in the rumen leaves little 22:6*n*-3 for absorption.

Biohydrogenation of PUFA is one of the important microbial processes in the rumen that has a major influence on FA composition of meat and milk. It is well documented that ruminal bacteria are responsible for most of the biohydrogenation process in the rumen (Jenkins *et al*., 2008), and it is thought to be a detoxification mechanism as PUFA are more toxic than SFA (Maia *et al*., 2010). Metabolism of linoleic (18:2*n*-6) and linolenic (18:3*n*-3) acids is well studied *in vivo* and *in vitro* and involves an initial isomerization step to yield a FA with a conjugated double bond (a pair of double bonds separated by one single bond). *In vitro* studies with mixed and pure rumen bacteria helped to construct the detailed biohydrogenation pathways of 18:2*n*-6 and 18:3*n*-3, and the identification of bacterial species involved in this process (Harfoot and Hazlewood, 1997; Shingfield *et al*., 2010b; Wallace *et al*., 2006). However, information on the biohydrogenation of 22:6*n*-3 is still lacking. Several reports show extensive metabolism of 22:6*n*-3 *in vivo* (Shingfield *et al*., 2010b, 2012b) and *in vitro* with mixed rumen cultures (AbuGhazaleh and Jenkins, 2004a; Vlaeminck *et al*., 2014). However, none of the studies identified bacterial species responsible for its metabolism. Maia *et al*. (2007) investigated PUFA metabolism of 26 predominant rumen bacterial species and found none of them able to metabolize 22:6*n*-3.

Sakurama et al. (2014) screened about 100 strains of anaerobic bacteria including some ruminal bacteria and found none of them metabolized 22:6*n*-3. Pure culture studies focusing on the main rumen hydrogenating bacteria, *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* also failed to successfully induce 22:6*n*-3 hydrogenation (Maia *et al*., 2007).

The aim of this study was to examine the metabolism of 22:6*n*-3 by the known biohydrogenating ruminal bacteria, *B. fibrisolvens* and *B. proteoclasticus*. To our knowledge, in previous experiments these bacterial species have been exposed to a single concentration of 22:6*n*-3 (50 µg/mL) which may be toxic for them (Maia *et al*., 2007). We used lower concentrations of 22:6*n*-3 in our experiments. Additionally, we modified the standard *Butyrivibrio* growth medium in an attempt to promote biohydrogenation. Experiments 1-5 were conducted using the growth medium containing autoclaved-uncentrifuged rumen fluid and experiments 6-8 were conducted using the growth medium containing autoclaved-centrifuged rumen fluid.

MATERIALS AND METHODS

Microorganisms and cultivation

B. fibrisolvens D1 (DSM 3071) and *B. proteoclasticus* P18 were selected for this study. *B. fibrisolvens* D1 was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and *B. proteoclasticus* P18 was obtained from the culture collection of the Rowett Institute of Nutrition and Health (University of Aberdeen, Bucksburn, Aberdeen AB21 9SB, UK). *Butyrivibrio* medium (DSMZ: medium 704) was slightly modified: VFA mixture, haemin and glycerol were omitted from the basic medium and L-cysteine-HCl was

used as the only reducing agent (0.5 g/l). The preparation method of rumen fluid and the rumen fluid/buffer ratio were also modified (Table 1).

Uncentrifuged-autoclaved (uRF) or centrifuged-autoclaved (cRF) rumen fluids were used for the growth media preparation. Rumen fluid was collected from 3 mature wethers, fitted with a ruminal cannula, fed grass hay and a commercial grain concentrate twice a day according to their maintenance requirements. To obtain the centrifuged rumen fluid, rumen fluid collected from cannulated wethers, was filtered through a sieve with a pore size of 1 mm and then fine particles were removed from the filtrate by centrifugation at 10,000 *g* for 20 min at 4 °C. The supernatant was sterilized by autoclaving for 20 min at 121 °C and stored frozen at -20 °C. The stored rumen fluid was thawed before use and any new precipitates formed were removed by centrifugation at 12,000 *g* for 15 min at 4 °C. For (uRF) rumen fluid, rumen fluid collected from cannulated wethers was filtered through a sieve with a pore size of 1 mm and autoclaved (20 min at 121 °C), and used without further processing.

In vitro incubations were carried out anaerobically at 39 °C under continuous shaking in Hungate-type tubes (16 mm dia., 125 mm long; Bellco Glass, Vineland, NJ, USA) containing 9.5 mL of medium and closed with screw caps fitted with butyl rubber septa (Chemglass Life Sciences, Vineland, NJ, USA), and autoclaved for 20 min at 121 °C. Inoculum volumes were 5% (v/v) of a fresh culture that was grown in medium 704 for 12-h ($OD_{600} \approx 1.7$). Growth was determined by measuring the culture density at 600 nm (Ultraspec10, Amersham Biosciences corp., Piscataway, NJ, USA). At the end of the experiment the incubations were stopped by placing the tubes in ice water, pH was measured (Hanna instruments, Temse, Belgium) and culture contents were sampled for VFA and long chain FA (LCFA) analysis.

Fatty acid solution

Fatty acid solutions were prepared by dispersing 200 mg of 22:6*n*-3 (Nu-check-Prep., Elysian, MN, USA) in 3.33 mL of a 0.06 M Tween-80 (Sigma Aldrich, St Louis, MO) solution. Then 0.25 mL of 3 M NaOH was added to obtain a clear solution. This solution was diluted with distilled water to achieve a final 22:6*n*-3 concentration of 10 mg/mL. The amount of Tween-80 and NaOH in all the tubes was kept the same by using a blank solution (prepared with Tween-80 and NaOH only). The required amount of the FA solution was added before autoclaving individually to each Hungate tube.

***In vitro* experiments**

Previous experiments performed with *Butyrivibrio* species in the medium containing cRF failed to hydrogenate 22:6*n*-3 (Maia *et al*., 2007, 2010). As such, experiments 1-5 (Table 1) were conducted using the growth medium containing uRF. In experiment 1, *B. fibrisolvens* was grown in the medium containing either 20 or 50% (v/v) uRF. The concentration of 22:6*n*-3 used (20 µg/mL) was lower than the previously reported value (50 µg/mL) (Maia *et al*., 2007, 2010). All treatments were conducted with 3 replicates.

Since we saw the 22:6*n*-3 disappearance by *B. proteoclasticus* in the medium containing 50% (v/v) uRF, additional experiments were conducted in this medium (Table 1). Influence of headspace gas (Exp. 2), incubation period (Exp. 3) and initial concentration of 22:6*n*-3 (Exp. 4) were studied in these experiments. *B. proteoclasticus* is the only stearic acid forming rumen bacteria so far identified. Formation of stearic acid (18:0) may be affected in the presence of 22:6*n*-3 as 22-

carbon FA are more toxic than the 18-C FA. As such influence of 18:2*n*-6 (40 µg/mL) on biohydrogenation of 22:6*n*-3 (0, 10 and 40 µg/mL) was studied in experiment 5. All treatments were performed in duplicate (analytical replicates) with inoculum from two different culture tubes (biological replicates).

Experiments 6-8 were conducted using cRF (Table 1). In experiment 6, *B. fibrisolvens* was grown with different concentrations of 22:6*n*-3 and growth (culture density) was monitored during the experiment. Lag time for the growth was determined based on the time point at which the increase in OD_{600} initiated. Linoleic acid (18:2*n*-6) was used as the positive control. Treatments were performed in duplicate (analytical replicates) with inoculum from three different culture tubes (biological replicates).

In experiment 7, we saw the disappearance of 22:6*n*-3 (20 µg/mL) by *B. proteoclasticus* in the medium containing 20% (v/v) cRF after 24-h incubation. Kinetics of this disappearance was studied in experiment 8 along with the growth of the bacteria by measuring increase in culture density (OD_{600}) at the time of tube withdrawal. Three analytical replicates were used for each treatment.

Table 1. Overview of the *in vitro* experiments conducted in this study.

Exp.	Autoclaved-rumen fluid/tube (%)	Bacteria[1]	22:6*n*-3 (µg/mL)	Gas phase[2]	Incubation period (h)
	Uncentrifuged (uRF)				
1	20 and 50	B. fibri	20	CO_2	0 and 48
2	50	B. proteo	20	CO_2, H_2 and N_2	0 and 48
3	50	B. proteo	20	H_2	0, 2, 4, 8, 12, 24 and 48
4	50	B. proteo	5, 10, 40 and 80	H_2	0 and 48
5[3]	50	B. proteo	10 and 40	H_2	0, 2, 4, 8, 12, 24 and 48
	Centrifuged (cRF)				
6	20 and 50	B. fibri	0.5, 1, 2, 4, 8, 16 and 32	CO_2	0 and 48
7	20	B. proteo	20	H_2	0 and 24
8	20	B. proteo	20	H_2	0, 2, 4, 8, 12 and 24

[1] Bacteria used in the experiment -B. fibri*: B. fibrisolvens* D1; B. proteo: *B. proteoclasticus* P18

[2] headspace gas phase

[3] The growth medium used in experiment 5 contained 40 µg/mL of 18:2*n*-6.

Analysis and calculation

For VFA analysis, 2 mL of incubation medium were collected and acidified with 200 µL of formic acid which contained the internal standard (10 mg of 2-ethyl butyric acid/mL formic acid). After 15 min centrifugation at 4 °C and 22,000 *g*, supernatant was filtered and an aliquot was transferred into a 1.5 mL glass vial. Samples were stored at 4 °C until VFA analysis using gas chromatography (HP 7890A, Agilent Technologies, Diegem, Belgium) equipped with a flame ionization (FID) detector detector and a Supelco Nukol capillary column (30 m × 0.25 mm × 0.25 µm, Sigma-Aldrich, Diegem, Belgium). The temperature program was as follows: 120 °C at injection for 0.2 min; increased at 10 °C/min until 180 °C and remained at this temperature for 3 min; injector temperature: 250 °C; detector temperature 255 °C. For this temperature program, 0.3 µL was injected with a split/splitless ratio of 25:1 using H_2 as carrier gas at 0.8 mL/min. VFA peaks were identified based on their retention times, compared to external standards (Sigma Aldrich, St Louis, MO).

The remainder of the content in the Hungate tubes after removal of 2 mL for VFA analysis (8 mL/tube) was freeze-dried for LCFA analysis and FA were methylated as described by Vlaeminck et al. (Vlaeminck *et al*., 2014). Analysis of the FA methyl esters (FAME) was carried out using a gas chromatograph (HP7890A, Agilent Technologies, Diegem, Belgium) using a SP-2560 column (75m x 0.18 mm, i.d. x 0.14 µm thickness, Supelco Analytical, Bellefonte, USA) and a FID detector. The temperature program was initially 70 °C for 2 min, increasing at 15 °C/min to 150 °C, followed by a second increase at 1 °C/ min up to 165 °C and holding for 12 min, followed by a third increase at 5 °C/min to 210 °C , held at 210 °C for 20 min, increased at 5 °C/min to 220 °C and held at 220 °C for 15 min. Inlet and detector

temperatures were 250 and 255 °C, respectively. The split ratio was 50:1. Hydrogen was used as the carrier gas at a flow rate of 1 mL/min. Identities of peaks were determined using mixtures of methyl ester standards (GLC463, Nu-Check-Prep., Inc., Elysian, MN, USA).

Quantification of FA was based on the area of the internal standard and on the conversion of peak areas to the weight of FA by a theoretical response factor for each FA (Ackman and Sipos, 1964; Wolff *et al*., 1995).

Structural analysis of fatty acid intermediates

Methyl esters not contained in commercially available standards were identified based on GC-MS analysis of DMOX derivatives prepared from FAME. Prior to preparation of DMOX derivatives, FAME were fractionated using Ag^+-SPE columns (750 mg/6 mL, Supelco, Bellefonte, PA, USA). Columns were activated with 4 mL acetone, followed by 4 mL hexane. The FAME of selected samples, dissolved in 1 mL hexane, were loaded on the column and eluted with hexane containing increasing amounts of acetone (v/v): 6 mL (99/1), 2 × 3 mL (96/4), 2 × 3 mL (90/10), 2 × 3 mL (0/100). This was followed by elution with acetone containing increasing amounts of acetonitrile (v/v): 2 × 3 mL (98/2), 2 × 3 mL (96/4), 2 × 3 mL (94/6), 2 × 3 mL (90/10) and 2 × 3 mL (80/20). All fractions were taken to dryness in a stream of N_2, dissolved in hexane and used for analysis of FAME by GC, as described above, and preparation of 4, 4-dimethyloxazoline (DMOX) derivatives.

DMOX derivatives of FA were prepared by using a modified procedure (Destaillats *et al*., 2005). Briefly, FAMEs were converted into DMOX derivatives with 500 µL 2-

amino-2-methyl-1-propanol under a nitrogen atmosphere at 170 °C overnight. DMOX derivatives were extracted twice with diethyl ether/n-hexane (1:1, v/v) and sodium chloride-saturated water. The organic layer was dried with anhydrous sodium sulphate for 1 h, followed by evaporation until dry under nitrogen. The DMOX derivatives were dissolved in hexane.

Identification of the DMOX derivatives of the biohydrogenation intermediates was based on electron impact ionisation spectra obtained by gas chromatography-mass spectrometry (GC-MS),using a gas chromatograph (Trace2D-GC,Thermo Electron Corporation, Waltham, MA, USA) coupled to a quadrupole mass detector (DSQII, Thermo Electron Corporation) under an ionisation voltage of 70 eV, using helium as carrier gas. The ion source and interface temperatures were maintained at 200 °C and 250 °C, respectively. The column was a SLB 5ms capillary column (60 m x 0.25 mm, i.d. x 0.25 µm thickness, Supelco Analytical, Bellefonte, USA). The electron impact ionization spectra obtained were used to locate double bonds based on atomic mass unit (amu) distances with an interval of 12 amu between the most intense peaks of clusters of ions containing n and n-1 carbon atoms, being interpreted as cleavage of the double bond between carbon n and n+1 in the fatty acid moiety.

Statistical analysis

All statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC). A least squares mean ANOVA in the GLM procedure using 22:6*n*-3 level (Exp. 1, 4, 5 and 6) or incubation time (Exp. 3 and 8) or headspace gas (Exp. 2) as factors was used to test time and treatment effects. The Tukey-Kramer test was performed

to assess which treatments differed in case of multiple (> 2) treatments. The differences among means with $P < 0.05$ were considered to be statistically significant.

RESULTS

22:6*n*-3 metabolism by *B. fibrisolvens* D1

Both cRF and uRF (Exp. 1 and 6), did not result in 22:6*n*-3 metabolism at any concentration. The effect of various concentrations of 22:6*n*-3 on growth, fermentation and biohydrogenation ability of *B. fibrisolvens* D1 (Exp. 6) in media containing 20% (v/v) cRF are summarized in Table 2.

Table 2. Effects of different concentrations of 22:6*n*-3 on growth, VFA production and biohydrogenation by *B. fibrisolvens* D1.

	Concentration of 22:6*n*-3 (µg/mL)			
	0	**8**	**16**	**32**
Lag phase (h)	< 3	< 3	4-5	> 48
OD_{600}[1]	1.13 ± 0.07[a]	1.22 ± 0.10[a]	1.04 ± 0.07[b]	ND[2]
Total VFA (µmol/tube)	93.0 ± 3.3 [a]	94.0 ± 6.2[a]	94.2 ± 4.9[a]	ND
Biohydrogenation	No	No	No	No

[1] Increase in OD_{600} after 48-h of incubation compared to initial OD_{600} at 0-h

[2] ND- Not determined as growth was not started till 48-h

Means with different superscripts are significantly different ($P < 0.05$)

No growth was observed till 48-h with the highest concentration of 22:6*n*-3 (32 µg/mL), whereas a considerably shorter lag phase (4-h) was observed with the same concentration of 18:2*n*-6 in the same medium. Optical density (OD_{600}) measured at stationary phase was lower ($P < 0.05$) at the concentration of 16 µg/mL compared to the lower concentrations (0 and 8 µg/mL). Total volatile fatty acid (VFA) production and VFA profile were not affected ($P > 0.05$) by the 22:6*n*-3 concentrations. The main fermentation products included butyrate and small amounts of acetate (about 96% and 4% of molar concentration of VFA respectively).

22:6*n*-3 metabolism by *B. proteoclasticus* P18

In the growth medium containing 50% (v/v) uRF, *B. proteoclasticus* P18 hydrogenated 22:6*n*-3 (Exp. 2). At the concentrations of 20 µg/mL (0.2 mg/tube), most of the 22:6*n*-3 was metabolized, leaving little residual 22:6*n*-3 (0.005±0.003 mg/tube) after 48-h of incubation. The type of headspace gas had no influence on the residual amount of 22:6*n*-3. Total VFA production and VFA profile were not affected ($P > 0.05$) by different gas phases (data not shown). The main fermentation products included butyrate and acetate (about 70% and 30% of molar concentration of VFA respectively).

Effect of length of incubation period on 22:6*n*-3 metabolism by *B. proteoclasticus* P18

The extent of 22:6*n*-3 metabolism in media containing 50% (v/v) uRF at different time points (Exp. 3) is shown in Figure 1 along with the VFA production at the respective time points. Metabolism of 22:6*n*-3 (20 µg/mL) initiated after 4-h and 80% of 22:6*n*-3

was hydrogenated between 4-h and 12-h of incubation. Accumulation of VFA initiated prior to the start of 22:6*n*-3 metabolism and 93% of VFA were produced within the first 12-h of the incubation. Both the rate of 22:6*n*-3 disappearance and VFA production slowed down after 12-h of incubation. Significant VFA production was not observed after 12-h, whereas hydrogenation of 22:6*n*-3 slowly continued. As a result, the amount of 22:6*n*-3 recovered after 48-h was lower ($P < 0.05$) than the amount recovered after 12 and 24-h of incubation.

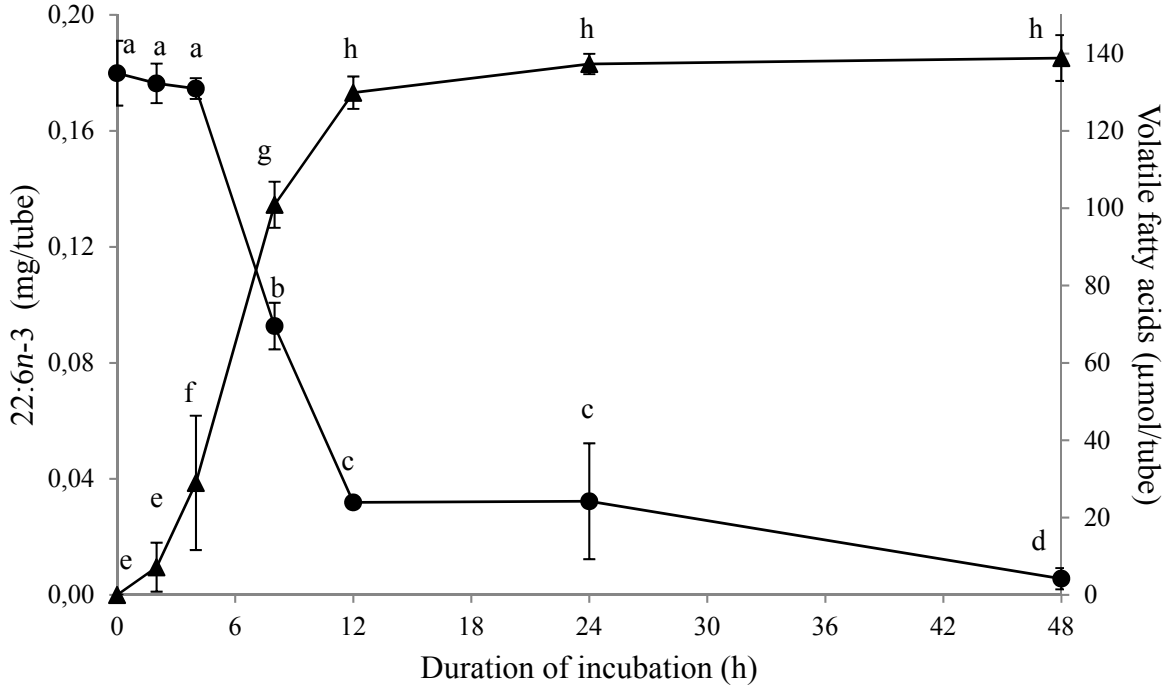

Figure 1: Metabolism of 22:6*n*-3 and VFA accumulation at different time points by *B. proteoclasticus* P18. Growth medium included 50% (v/v) of uRF. Hydrogen was used as the headspace gas. ● Residual 22:6*n*-3 in the tube and ▲ VFA accumulated. Results are means and SD from 2 biological replicates, each of which had 2 analytical replicates. For residual 22:6*n*-3 and accumulated VFA, data points with different letters are significantly different ($P < 0.05$).

Detailed analysis of chromatograms did not provide evidence of 22:0 formations during metabolism of 22:6*n*-3. Peaks appearance at different time points did not show accumulation of conjugated 22:6 products. The intermediates produced during the 48-h incubation comprised about 12 different FA (Table 3) and characteristic ion fragments are presented in Table 4. During the early hours of incubation (< 8-h), 4 new peaks (i.e. peaks which were not present in the 0-h incubation) appeared (Figure 2a: peaks 1-4). Of these, peak 3 was the most abundant and the molecular ion at m/z = 383 and the loss of the terminal methyl group (m/z = 368) confirmed the docosapentaenoic acid structure. The odd numbered ion fragment at m/z = 153 is an indication for the location of a Δ5 double bond (Christie, 1998). Gaps of 12 amu between m/z = 208 and 220; 248 and 260; 288 and 300 and between 328 and 340 confirmed the location of ethylenic double bonds in position Δ10, Δ13, Δ16 and Δ19 (Table 4). Peak 1 showed the same retention time as 22:5*n*-3 present in the FAME standard and ion fragments at 168 and 180, 208 and 220, 248 and 260, 288 and 300, and 328 and 340 allowed us to identify this peak as 22:5*n*-3 (Yu *et al*., 1989). A final docosapentaenoic acid (peak 2) was identified as Δ4, Δ10, Δ13, Δ16, Δ19-22:5 based on the molecular ion at m/z = 383, gaps of 12 amu between m/z = 208 and 220; 248 and 260; 288 and 300 and between 328 and 340 and the double bond in position 4 is defined by the fingerprint ion at m/z = 139 and 152 (Dobson and Christie, 1996). One docosatetraenoic acid was identified as Δ10, Δ13, Δ16, Δ19-22:4 (peak 4) based on the molecular ion at m/z = 385 and gaps of 12 amu between m/z = 210 and 222; 250 and 262; 290 and 302 and between 330 and 342. During the further incubation, the abundance of these peaks gradually decreased and were accompanied with the appearance of four 22:3 FA (peaks 5-8: Figure 2a) and four 22:2 FA (peaks 9-12: Figure 2a). Peaks 5 and 7 showed a similar mass spectra and

were identified as Δ13, Δ16, Δ20-22:3. Ion fragments separated by 12 amu gaps located the double bonds at Δ13, Δ16 and Δ20 with a prominent ion at m/z 332 confirming the presence of allylic bonds at Δ16 and Δ20. Peak 6 showed the same retention time as 22:3n-3 present in the FAME standard and double bond positions were confirmed by ion fragments separated by 12 amu gaps (Table 4). Peak 8 was identified as Δ11, Δ16, Δ19-22:3 based on ion fragments at 224 and 236, 292 and 304, and 332 and 344. Peaks 9 and 11 showed a similar mass spectra and were identified as Δ16, Δ20-22:2. Ion fragments separated by 12 amu gaps located the double bonds at Δ16 and Δ20 (Table 4) with a prominent ion at m/z 334 confirming the presence of allylic bonds at Δ16 and Δ20. Peak 6 was Δ16, Δ19-22:2 (ion fragments separated by 12 amu gaps at 294 and 306, and 334 and 346) and the prominent ion at m/z 306 combined ion fragments separated by 12 amu gaps at 266 and 278, and 320 and 332 allowed us to identify peak 12 as Δ14, Δ18-22:2.

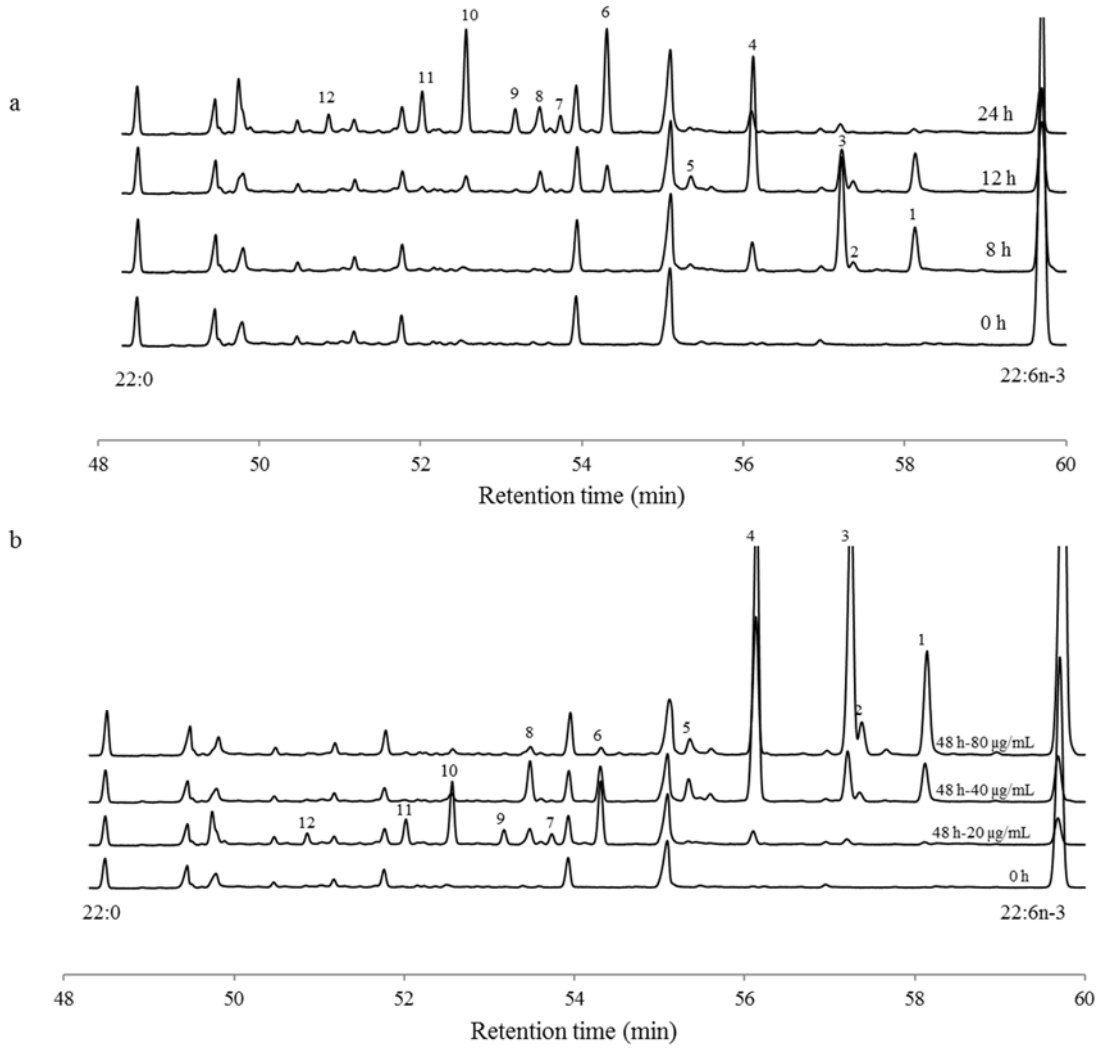

Figure 2. Partial chromatogram with peaks representing fatty acids from 22:0 to 22:6*n*-3. These chromatograms obtained from a) 0, 8, 12 and 24-h incubations with 22:6*n*-3 (20 µg/mL) and b) 48-h incubations with 22:6n-3 (20, 40 and 80 µg/mL). In both cases *B. proteoclasticus* P18 had grown in the media containing 50% (v/v) uncentrifuged-autoclaved rumen fluid (uRF). Peaks visible at 0-h represent the fatty acids present in the rumen fluid and peaks numbers (1-12) represent new peaks formed during the 22:6*n*-3 metabolism.

Table 3. Amount of metabolized 22:6*n*-3 (µg/tube) recovered in FAME peaks. Incubation was performed in growth medium containing uncentrifuged-autoclaved rumen fluid (uRF; 50% v/v) in the presence of 22:6*n*-3 (20 µg/mL) with *B. proteoclasticus* P18. Tubes were withdrawn at 0, 2, 4, 8, 12, 24 and 48-h incubation to study the extent of 22:6*n*-3 biohydrogenation. Peak numbers correspond to peaks in Figure 2.

Peaks		**Incubation duration (h)**				
		4	**8**	**12**	**24**	**48**
1	Δ7, Δ10, Δ13, Δ16, Δ19-22:5	2.18 ± 0.66	16.88 ± 0.94	16.38 ± 2.09	3.39 ± 1.71	2.93 ± 1.04
2	Δ4, Δ10, Δ13, Δ16, Δ19-22:5	-	3.36 ± 0.38	4.19 ± 1.04	1.07 ± 0.34	0.43 ± 0.58
3	Δ5, Δ10, Δ13, Δ16, Δ19-22:5	3.92 ± 1.29	44.38 ± 1.59	16.60 ± 5.80	7.40 ± 3.51	3.68 ± 2.42
4	Δ10, Δ13, Δ16, Δ19-22:4		9.42 ± 2.55	55.40 ± 3.00	11.27 ± 2.34	19.69 ± 2.41
5	Δ13, Δ16, Δ20-22:3		3.12 ± 0.70	8.17 ± 1.99	1.73 ± 0.11	3.63 ± 1.62
6	Δ13, Δ16, Δ19-22:3		-	9.91 ± 1.90	33.05 ± 4.66	36.02 ± 5.88
7	Δ13, Δ16, Δ20-22:3		-	0.69 ± 0.86	4.91 ± 0.38	7.24 ± 0.49
8	Δ11, Δ16, Δ19-22:3		-	9.10 ± 0.99	10.69 ± 1.66	8.77 ± 1.22
9	Δ16, Δ20-22:2		-	0.72 ± 0.90	7.45 ± 0.49	10.75 ± 0.76
10	Δ16, Δ19-22:2		-	6.31 ± 1.67	31.24 ± 2.96	37.31 ± 4.61
11	Δ16, Δ20-22:2		-	1.34 ± 0.93	11.75 ± 1.25	16.69 ± 1.46
12	Δ14, Δ18-22:2		-	-	5.03 ± 0.48	7.49 ± 0.56

Note: peaks with the same number of double bound at the same delta (Δ) position differ in *cis/trans* configuration of at least one double bond (peaks 5 and 7; and peaks 9 and 11)

Table 4. Characteristic ion fragments recorded during gas-chromatography mass-spectrometry analysis of 4,4-dimethyloxazoline derivatives of newly formed fatty acids during biohydrogenation of 22:6*n*-3 by *Butyrivirbio proteoclasticus*. Peak numbers correspond to peaks in Figure 2.

Peak	Fatty acid	Characteristic ion fragments (m/z, relative intensity)
1	Δ7, Δ10, Δ13, Δ16, Δ19-22:5	113 (64), 126 (100), 168 (9), 180 (24), 194 (21), 208 (11), 220 (6), 234 (12), 248 (16), 260 (6), 274 (12), 288 (6), 300 (5), 314 (10), 328 (3), 340 (2), 354 (2), 368 (4), 383 (9)
2	Δ4, Δ10, Δ13, Δ16, Δ19-22:5	113 (100), 126 (17), 139(15), 152 (49), 166 (52), 180 (15), 194 (25), 208 (4), 220 (5), 234 (14), 248 (6), 260 (7), 274 (1), 288 (8), 300 (4), 314 (12), 328 (3), 340 (2), 354 (4), 368 (4), 383 (11)
3	Δ5, Δ10, Δ13, Δ16, Δ19-22:5	113 (100), 126 (21), 153 (18), 166 (4), 180 (14), 194 (9), 208 (1), 220 (3), 234 (8), 248 (2), 260 (1), 274 (3), 288 (2), 300 (1), 314 (4), 328 (1), 340 (1), 354 (1), 368 (2), 383 (3)
4	Δ10, Δ13, Δ16, Δ19-22:4	113 (88), 126 (100), 168 (11), 182 (10), 196 (13), 210 (5), 222 (4), 236 (6), 250 (13), 262 (4), 276 (12), 290 (24), 302 (8), 316 (25), 330 (8), 342 (5), 356 (6), 370 (8), 385 (28)

Table 4. (*Continued*)

Peak	Fatty acid	Characteristic ion fragments (m/z, relative intensity)
5/7	Δ13, Δ16, Δ20-22:3	113 (64), 126 (57), 168 (9), 182 (6), 196 (4), 210 (4), 224 (4), 238 (7), 252 (3), 264 (1), 278 (8), 292 (7), 304 (3), 318 (3), 332 (100), 346 (2), 358 (3), 372 (8), 387 (17)
6	Δ13, Δ16, Δ19-22:3	113 (100), 126 (100), 168 (14), 182 (11), 196 (8), 210 (7), 224 (7), 238 (10), 252 (4), 264 (2), 278 (7), 292 (12), 304 (5), 318 (22), 332 (16), 344 (8), 358 (13), 372 (15), 387 (42)
8	Δ11, Δ16, Δ19-22:3	113 (80), 126 (100), 168 (18), 182 (11), 196 (9), 210 (11), 224 (7), 236 (4), 250 (3), 264 (9), 278 (45), 292 (2), 304 (5), 318 (14), 332 (6), 344 (5), 358 (3), 372 (15), 387 (21)
9/11	Δ16, Δ20-22:2	113 (100), 126 (56), 168 (11), 182 (7), 196 (4), 210 (3), 224 (3), 238 (6), 252 (3), 266 (4), 280 (10), 294 (8), 306 (1), 320 (2), 334 (75), 348 (2), 360 (2), 374 (6), 389 (11)
10	Δ16, Δ19-22:2	113 (100), 126 (69), 168 (12), 182 (8), 196 (5), 210 (4), 224 (6), 238 (8), 252 (5), 266 (6), 280 (10), 294 (3), 306 (2), 320 (13), 334 (11), 346 (9), 360 (10), 374 (19), 389 (34)
12	Δ14, Δ18-22:2	113 (100), 126 (83), 168 (14), 182 (15), 196 (9), 210 (6), 224 (7), 238 (13), 252 (7), 266 (7), 278 (3), 292 (14), 306 (80), 320 (<1), 332 (5), 346 (12), 360 (25), 374 (14), 389 (26)

Note: peaks with the same number of double bound at the same delta (Δ) position differ in *cis/trans* configuration of at least one double bond (peaks 5 and 7; and peaks 9 and 11).

In experiments conducted in media containing cRF (20% v/v), *B. proteoclasticus* hydrogenated 22:6*n*-3 at the concentration of 20 µg/mL (Exp. 7 and 8). After 24-h of incubation, little residual 22:6*n*-3 was left in the tube (0.006 ± 0.0003 mg/tube). Figure 3 shows the kinetics of 22:6*n*-3 disappearance along with OD_{600} measured at the respective time points (Exp. 8). Metabolism of 22:6*n*-3 initiated after 4-h and 93% of 22:6*n*-3 was hydrogenated between 4-h and 12-h of incubation. Growth of *B. proteoclasticus* (measured as the increase in the OD_{600}) initiated prior to the start of 22:6*n*-3 metabolism and exponentially increased between 4-h and 12-h. The products formed during the biohydrogenation of 22:6*n*-3 (Table 5) were identical as those described before (Exp. 3).

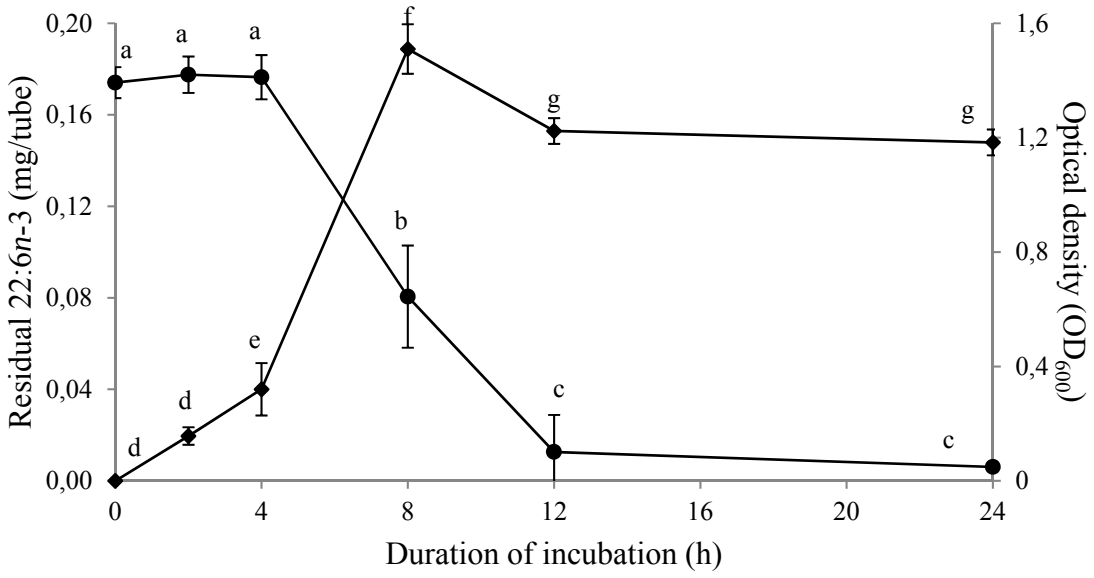

Figure 3: Metabolism of 22:6*n*-3 and optical density measurements at different time points by *B. proteoclasticus* P18. Growth medium included 20% (v/v) of cRF. Hydrogen was used as the headspace gas. ● Residual 22:6n-3 in the tube ♦ Optical density (OD_{600}) measured at respective time points. Results are means and SD from 3 replicates. For residual 22:6*n*-3 and OD_{600} measured, data points with different letters are significantly different ($P < 0.05$).

Table 5. Amount of metabolized 22:6*n*-3 (µg/tube) recovered in FAME peaks. Incubation was performed in growth medium containing cRF (20% v/v) in the presence of 22:6*n*-3 (20 µg/mL) with *B. proteoclasticus* P18. Tubes were withdrawn at 0, 2, 4, 8, 12 and 24-h incubation to study the extent of 22:6*n*-3 biohydrogenation. Peak numbers correspond to peaks in Figure 2.

Peaks		**Incubation duration (h)**			
		4	**8**	**12**	**24**
1	Δ7, Δ10, Δ13, Δ16, Δ19-22:5	5.51 ± 0.59	19.55 ± 2.82	13.72 ± 3.93	-
2	Δ4, Δ10, Δ13, Δ16, Δ19-22:5	-	3.68 ± 1.22	5.21 ± 3.88	-
3	Δ5, Δ10, Δ13, Δ16, Δ19-22:5	4.41± 1.10	53.32 ± 8.58	28.37 ± 39.9	-
4	Δ10, Δ13, Δ16, Δ19-22:4		11.34 ± 3.05	91.90 ± 40.6	26.41 ± 36.1
5	Δ13, Δ16, Δ20-22:3		6.89 ± 1.25	11.86 ± 3.27	9.34 ± 5.26
6	Δ13, Δ16, Δ19-22:3		-	10.26 ± 5.80	38.69 ± 3.97
7	Δ13, Δ16, Δ20-22:3		-	0.36 ± 0.32	6.75 ± 5.59
8	Δ11, Δ16, Δ19-22:3		-	10.10 ± 6.52	15.73 ± 16.6
9	Δ16, Δ20-22:2		-	0.34 ± 0.30	7.36 ± 7.36
10	Δ16, Δ19-22:2		-	1.38 ± 1.21	26.68 ± 11.4
11	Δ16, Δ20-22:2		-	-	10.09 ± 10.8
12	Δ14, Δ18-22:2		-	-	6.69 ± 5.96

Note: peaks with the same number of double bound at the same delta (Δ) position differ in *cis/trans* configuration of at least one double bond (peaks 5 and 7; and peaks 9 and 11).

Effect of initial concentration of 22:6*n*-3 on 22:6*n*-3 metabolism by *B. proteoclasticus* P18

The initial concentration of 22:6*n*-3 influenced the extent of its metabolism. Extensive metabolism of 22:6*n*-3 was observed at lower concentrations (5, 10 and 20 µg/mL). Addition of 5, 10 and 20 µg/mL 22:6*n*-3 resulted in the accumulation of 22:2 and 22:3 FA and represented > 80% of 22:6*n*-3 that was metabolized during the 48-h incubation (Table 6). This implies that the initial 22:5 and 22:4 FA were further hydrogenated. An increase in the initial concentration of 22:6*n*-3 increased the amount of 22:6*n*-3 metabolized during 48-h incubation period, but only up to a concentration of about 40 µg/mL (Figure 4). Although in absolute amounts, more 22:6*n*-3 disappeared at higher 22:6*n*-3 concentrations, analysis of the chromatograms showed that extensive biohydrogenation of intermediate products was inhibited (Table 6 and Figure 4). At the initial concentration of 40 µg/mL, the residual 22:6*n*-3 retrieved from the tubes after 48-h incubation was 9% of the initial amount. Under this condition, 65% of the metabolized 22:6*n*-3 accumulated as a single compound identified as Δ10, Δ13, Δ16, Δ19-22:4. Further conversion of this product seemed to be inhibited. When the initial 22:6*n*-3 concentration was increased to 80 µg/mL, the residual 22:6*n*-3 retrieved from the tubes after 48-h of incubation was 45% of the initial amount and > 90% of the metabolized 22:6*n*-3 accumulated as 22:4 and 22:5 FA. Subsequent biohydrogenation of these products seemed completely inhibited at this concentration.

Total VFA production was not affected by the initial concentration of 22:6*n*-3 except for the highest concentration (80 µg/mL). At the highest initial concentration (80 µg/mL) VFA production after 48-h was higher ($P < 0.05$) compared to the lower

concentrations (16.4 ± 0.5 and 15.1 ± 0.5 µmol/mL respectively), but the VFA profile was not affected ($P > 0.05$).

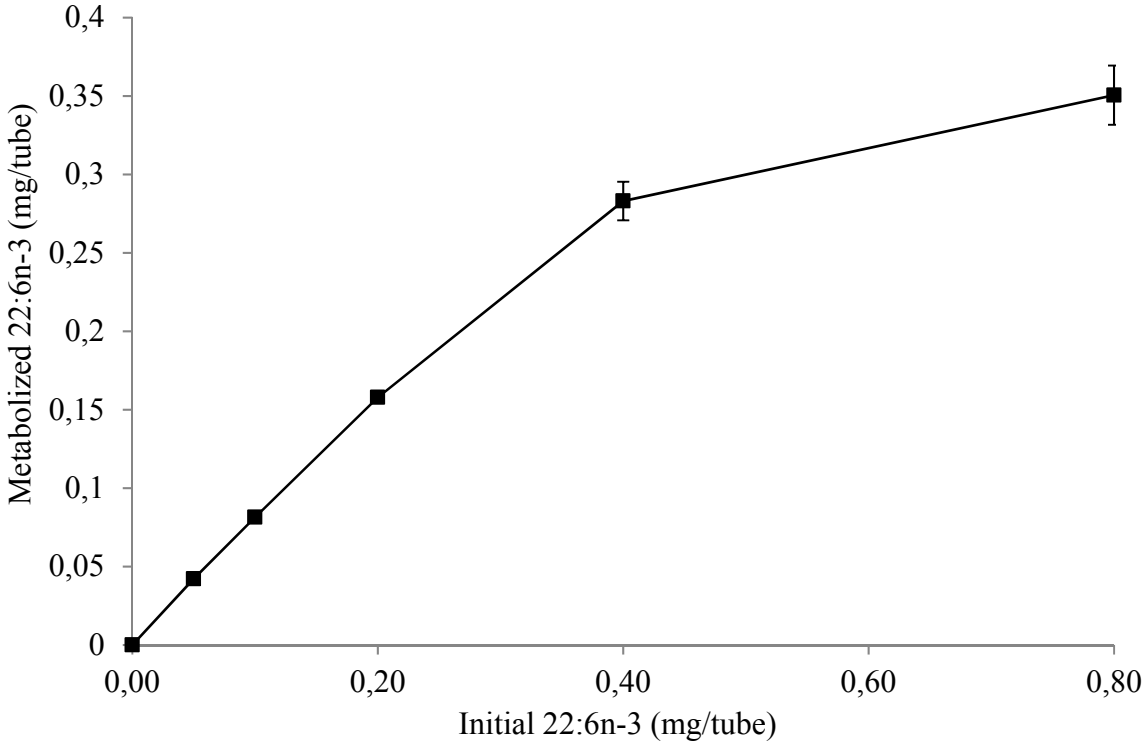

Figure 4: Effect of initial concentration of 22:6*n*-3 (5 - 80 µg/mL) on its metabolism by *B. proteoclasticus* P18. Incubations were performed in growth medium containing 50% (v/v) of autoclaved-uncentrifuged rumen fluid for 48-h.

Table 6. Amount of metabolized 22:6*n*-3 (µg/tube) recovered in FAME peaks. Incubation was performed in growth medium containing autoclaved-uncentrifuged rumen fluid (50% v/v) in the presence of 22:6*n*-3 (5, 10, 20, 40 and 80 µg/mL) with *B. proteoclasticus* P18. Tubes were withdrawn at 48-h of incubation to study the extent of 22:6n-3 biohydrogenation. Peak numbers correspond to peaks in Figure 2.

		Initial concentration of 22:6*n*-3 (µg/tube)				
Peaks		**50**	**100**	**200**	**400**	**800**
1	Δ7, Δ10, Δ13, Δ16, Δ19-22:5	0.63 ± 0.75	1.15 ± 0.28	2.93 ± 1.04	25.14 ± 3.03	77.118 ± 1.30
2	Δ4, Δ10, Δ13, Δ16, Δ19-22:5	-	-	0.43 ± 0.58	5.08 ± 1.05	21.42 ± 0.69
3	Δ5, Δ10, Δ13, Δ16, Δ19-22:5	0.87 ± 1.13	1.11 ± 0.96	3.68 ± 2.42	29.16 ± 3.92	177.29 ± 5.04
4	Δ10, Δ13, Δ16, Δ19-22:4	5.26 ± 1.98	8.14 ± 2.11	19.69 ± 2.41	208.41 ± 14.7	78.81 ± 7.61
5	Δ13, Δ16, Δ20-22:3	0.41 ± 0.82	1.17 ± 0.82	3.63 ± 1.62	15.80 ± 2.34	12.05 ± 1.17
6	Δ13, Δ16, Δ19-22:3	5.82 ± 0.77	14.26 ± 0.62	36.02 ± 5.88	22.22 ± 3.63	3.48 ± 1.19
7	Δ13, Δ16, Δ20-22:3	2.84 ± 0.46	5.02 ± 0.20	7.24 ± 0.49	-	-
8	Δ11, Δ16, Δ19-22:3	2.33 ± 0.86	4.26 ± 0.66	8.77 ± 1.22	26.98 ± 3.47	5.61 ± 0.81
9	Δ16, Δ20-22:2	4.14 ± 0.53	7.14 ± 0.39	10.75 ± 0.76	-	-
10	Δ16, Δ19-22:2	7.81 ± 0.81	15.92 ± 0.87	37.31 ± 4.61	5.53 ± 1.24	3.09 ± 1.05
11	Δ16, Δ20-22:2	8.83 ± 0.63	12.94 ± 0.59	16.69 ± 1.46	-	-
12	Δ14, Δ18-22:2	3.34 ± 0.65	5.09 ± 0.35	7.49 ± 0.56	-	-

Note: peaks with the same number of double bound at the same delta (Δ) position differ in *cis/trans* configuration of at least one double bond (peaks 5 and 7; and peaks 9 and 11).

Effect of presence of 22:6*n*-3 on 18:2*n*-6 metabolism by *B. proteoclasticus* P18

Table 7 shows the conjugated linoleic acids (CLA), vaccenic acid (VA; *trans*-11 18:1) and stearic acid (SA;18:0) formation from 40 µg/mL 18:2*n*-6 in presence of 0 (control), low (10 µg/mL) and high (40 µg/mL) 22:6*n*-3, along with VFA formation and residual 22:6*n*-3. The formation of CLA was observed as soon as inoculation was made. Although formation of CLA and VA were not affected ($P > 0.05$), conversion of VA into SA (18:0) was retarded ($P > 0.05$) in the presence of 22:6*n*-3 and this effect was dependent on 22:6*n*-3 concentration. Volatile fatty acids production was not initiated until all the 18:2*n*-6 had been metabolized and converted into VA regardless of presence of high or low 22:6*n*-3. Both 18:0 formation and 22:6*n*-3 metabolism began after the initiation of VFA production. In the control tubes, 18:0 formation started at about 4-h as soon as VFA production began and this conversion was rapid thereafter (Table 7). In the presence of low 22:6*n*-3 (10 µg/mL), *B. proteoclasticus* P18 initiated 22:6*n*-3 metabolism around 4-h, ahead of 18:0 formation (around 8-h of incubation). As a result, a small lag phase was observed in 18:0 formation after the initiation of VFA production. In presence of high 22:6*n*-3 (40 µg/mL), 18:0 formation was delayed to 12-h and this conversion was slower thereafter.

Table 7. Linoleic acid metabolism (mg/tube) in presence of 22:6*n*-3 by *B. proteoclasticus* P18. Incubation was performed in growth medium containing 40 µg/mL (0.4 mg/tube) of 18:2*n*-6 in presence of 0, 10 µg/mL (Low: 0.1 mg/tube) and 40 µg/mL (High: 0.4 mg/tube) 22:6*n*-3. Growth medium included 50% (v/v) of uRF and H_2 gas was the headspace gas. Value-presented in this table included the fatty acids and VFA of uRF used to prepare the growth medium.

		Incubation duration (h)						
	22:6*n*-3	**0**	**2**	**4**	**8**	**12**	**24**	**48**
18:2*n*-6	0	0.27 ± 0.006^{a}	0.09 ± 0.014^{b}	0.08 ± 0.009^{bc}	0.08 ± 0.002^{bc}	0.09 ± 0.012^{b}	0.06 ± 0.001^{c}	0.05 ± 0.002^{c}
(mg/tube)	Low	0.25 ± 0.007^{a}	0.09± 0.001^{bc}	0.09 ± 0.002^{bc}	0.09 ± 0.001^{bc}	0.10 ± 0.003^{b}	0.07 ± 0.006^{c}	0.06 ± 0.002^{c}
	High	0.28 ± 0.099^{a}	0.11 ± 0.002^{b}	0.10 ± 0.011^{bc}	0.10 ± 0.004^{b}	0.10 ± 0.006^{b}	0.07 ± 0.006^{c}	0.06 ± 0.005^{c}
CLA[1]	0	0.07 ± 0.005^{a}	0.02 ± 0.007^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}
(mg/tube)	Low	0.10 ± 0.007^{a*}	0.02 ± 0.004^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}
	High	0.11 ± 0.073^{a}	0.02 ± 0.003^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}
VA[2]	0	0.05 ± 0.005^{a}	0.27 ± 0.026^{b}	0.21 ± 0.011^{c}	0.07 ± 0.003^{d}	0.06 ± 0.005^{d}	0.06 ± 0.003^{d}	0.04 ± 0.008^{a}
(mg/tube)	Low	0.05 ± 0.005^{a}	0.30 ± 0.010^{b*}	0.29 ± 0.015^{c*}	0.10 ± 0.007^{d*}	0.07 ± 0.003^{e}	0.07 ± 0.004^{e}	0.07 ± 0.004^{e*}
	High	0.05 ± 0.026^{a}	0.29 ± 0.005^{b*}	0.28 ± 0.005^{b*}	0.19 ± 0.005^{c**}	0.11 ± 0.008^{d*}	0.13 ± 0.010^{d*}	0.10 ± 0.002^{d**}

Table 7. (*Continued*)

		Incubation duration (h)						
	22:6*n*-3	**0**	**2**	**4**	**8**	**12**	**24**	**48**
18:0	0	0.50 ± 0.011^{a}	0.51 ± 0.041^{a}	0.58 ± 0.014^{b}	0.74 ± 0.056^{c}	0.73 ± 0.020^{c}	0.71 ± 0.016^{c}	0.73 ± 0.019^{c}
(mg/tube)	Low	0.53 ± 0.019^{a}	0.53 ± 0.009^{a}	0.53 ± 0.022^{a*}	0.67 ± 0.019^{b*}	0.69 ± 0.018^{bc*}	0.72 ± 0.010^{c}	0.73 ± 0.010^{c}
	High	0.53 ± 0.006^{a}	0.53 ± 0.003^{a}	0.52 ± 0.017^{a*}	0.54 ± 0.015^{a**}	0.58 ± 0.015^{b**}	0.65 ± 0.017^{c*}	0.68 ± 0.007^{c*}
VFA[3]	0	434.1 ± 4.79^{a}	436.4 ± 7.77^{a}	469.6 ± 16.80^{b}	541.7 ± 12.61^{c}	545.7 ± 8.57^{c}	585.2 ± 30.61^{d}	567.6 ± 15.93^{d}
(μmol/tube)	Low	434.0 ± 9.61^{a}	437.7 ± 6.01^{a}	460.6 ± 3.09^{b}	527.9 ± 6.54^{cd}	542.9 ± 4.34^{de}	557.0 ± 7.23^{e*}	551.4 ± 5.35^{e}
	High	449.1 ± 16.6^{a}	433.0 ± 8.91^{a}	468.5 ± 21.71^{b}	531.8 ± 8.25^{c}	549.7 ± 6.14^{cd}	562.1 ± 17.97^{d*}	559.4 ± 2.82^{d}
22:6*n*-3	Low	0.07 ± 0.005^{a}	0.07 ± 0.002^{a}	0.06 ± 0.002^{b}	0.02 ± 0.001^{c}	0.02 ± 0.001^{c}	0.02 ± 0.003^{c}	0.01 ± 0.000^{d}
(mg/tube)	High	0.31 ± 0.018^{a}	0.30 ± 0.012^{a}	0.29 ± 0.011^{a}	0.17 ± 0.002^{b}	0.11 ± 0.004^{c}	0.09 ± 0.012^{c}	0.06 ± 0.006^{d}

[1]CLA: conjugated linoleic acid; [2]VA: vaccenic acid; [3]VFA: volatile fatty acids.

For each fatty acids, subscript * represents different (P < 0.05) compared to 0 mg/tube 22:6*n*-3 and ** represents different (P < 0.05) compared to 0 and 0.1 mg/tube 22:6*n*-3 at the respective time point.

Within each row means with different superscripts are significantly different (P < 0.05).

DISCUSSION

Butyrivibrio species are a genetically and functionally diverse group of bacteria present in gastrointestinal systems (Polan *et al*., 1964; Wallace *et al*., 2006). Based on the mechanism of butyrate formation, this group can be classified into two subgroups: vaccenic acid-producing (low butyrate kinase activity) and stearic acid-producing (high butyrate kinase activity). Accordingly, *B. fibrisolvens* and *B. proteoclasticus* belong to the vaccenic acid-producing and stearic acid-producing groups respectively (Paillard *et al*., 2007). *B. fibrisolvens* D1 and *B. proteoclasticus* P18 were chosen for this study as a representative from each group. However, the type species *B. fibrisolvens* D1 showed high butyrate kinase activity which is atypical to the majority of *B. fibrisolvens* isolates (Forster *et al*., 1996).

Previous studies carried out with *B. fibrisolvens* and *B. proteoclasticus* in M2 medium failed to show hydrogenation of 22:6*n*-3 (Maia *et al*., 2007, 2010). The reason for this is probably the high concentration of 22:6*n*-3 used in these studies (50 µg/mL) which may have affected the growth of the bacteria. Wallace et al. (2006) showed growth to be a prerequisite for *B. proteoclasticus* P18 in order to form stearic acid (18:0) from 18:2*n*-6. If this is also true for 22:6*n*-3 biohydrogenation, failure to grow when 22:6*n*-3 is present in previous experiments (Maia *et al*., 2007, 2010) possibly explains the absence of 22:6*n*-3 biohydrogenation. To reduce the inhibitory effect of 22:6*n*-3 on growth, the concentration of 22:6*n*-3 in the medium can be lowered or substances which lower the toxicity of 22:6*n*-3 should be included in the medium. Harfoot *et al*. (1973) showed that biohydrogenation was stimulated by rumen fluid particles, enabling FA biohydrogenation by ruminal bacteria. As showed in Chapter 2 of this

thesis, addition of uncentrifuged-autoclaved rumen fluid (uRF) stimulated the biohydrogenation of 22:6*n*-3 in mixed cultures of rumen bacteria. Hence, we used uRF to prepare the growth medium. Additionally, we used a lower concentration of 22:6*n*-3 than the concentration reported previously (50 µg/mL) (Maia *et al*., 2007, 2010).

B. fibrisolvens grew at low concentrations of 22:6*n*-3 without metabolizing it. Maia et al. (2010) showed that growth of *B. fibrisolvens* JW11 was not initiated until all 18:2*n*-6 was metabolized and converted to VA, such phenomenon was not observed with 22:6*n*-3. The failure of *B. fibrisolvens* D1 to metabolize 22:6*n*-3 in the current experiment might indicate they do not possess the necessary enzymes for 22:6*n*-3 biohydrogenation. However, further studies are warranted as *B. fibrisolvens* D1 is atypical to other *B. fibrisolvens* in general.

In contrast with previous reports (Maia *et al*., 2007), we found *B. proteoclasticus* P18 is able to hydrogenate 22:6*n*-3. To our knowledge, this is the first report demonstrating 22:6*n*-3 biohydrogenation by a pure bacterial species. It should be noted that in the current experiment with cRF, the initial concentration of 22:6*n*-3 was 20 µg/mL well below the concentration used in the study of Maia et al. (2007). This lower level of 22:6*n*-3 might explain the contrasting results found in the current study and the study of Maia et al. (2007).

Biohydrogenation of 22:6*n*-3 by *B. proteoclasticus* P18 in media containing cRF (20% v/v), is advantageous as we could follow the growth of the bacteria during the time course of the incubation.

Temporal changes in 22:6*n*-3, VFA and OD_{600} during the course of the incubation might indicate *B. proteoclasticus* must be growing to biohydrogenate 22:6*n*-3, with little transformation occurring during the stationary phase. Such observations were also made when demonstrating the capability of *B. proteoclasticus* P18 to form 18:0 from 18:2*n*-6 (Wallace *et al*., 2006). Stationary-phase bacteria were reported to be much less active in hydrogenation of 18:2*n*-6 than growing cells (Kim *et al*., 2000; Wallace *et al*., 2006). This was explained by a limited supply of reducing equivalents once the energy sources in the medium had been depleted (Wallace *et al*., 2006) as the conversion of CLA to VA is NADH-dependent (Hunter *et al*., 1976). It seems probable that a similar mechanism explains the variation in rate of disappearance of 22:6*n*-3 during the course of the incubation period. It is however not clear whether growth or activity per se is needed for biohydrogenation, or that the association of disappearance of 22:6n-3, VFA and OD_{600} is merely a reflection of changes in the culture closely associated with growth and activity (e.g. biomass production, redox potential).

The metabolism of 22:6*n*-3 resulted in the appearance of numerous compounds eluting during GC analysis between 22:0 and 22:6*n*-3. It has been speculated before that ruminal hydrogenation of 22:6*n*-3 yields intermediates with 5 or 6 double bonds, containing at least one *trans* double bond (Jenkins *et al*., 2008). The position of the double bonds in the most abundant 22:5 isomer (Δ5, Δ10, Δ13, Δ16, Δ19-22:5) suggest the formation of this intermediate involves izomerisation of the cis-4 double bond and reduction of the cis-7 double bond (Kairenius *et al*., 2011). Initial izomerisation of the cis-4 double bond of 22:6*n*-3 would result in the formation of a conjugated product (Δ5, Δ7, Δ10, Δ13, Δ16, Δ19-22:6). However, in the present

series of experiments, no accumulation of products eluting in the GC chromatogram with a retention time greater than 22:6*n*-3 was observed, a region where conjugated isomers of 22:6 would be expected to elute with the polar column used in the present study. The lack of accumulation of a conjugated FA might indicate they are transient products which did not accumulate at the time of sampling. Alternatively, this might also indicate the initial product of 22:6*n*-3 metabolism is not a conjugated FA. The accumulation of 22:5 isomers produced during the initial stages of 22:6*n*-3 biohydrogenation was transient, when the initial 22:6*n*-3 concentration was low, and these isomers were subsequently hydrogenated to more saturated 22:4, 22:3 and 22:2 isomers. The existence of two isomers with identical double bond position (peak 5 and 7 as Δ13, Δ16, Δ20-22:3 and peak 9 and 11 as Δ16, Δ20-22:2) indicates double bonds in the *trans* configuration must be present. It is unclear to what extent the increases in polyenoic *trans* fatty acids may offset some of the expected benefits from the enrichment of 22:6*n*-3 in ruminant derived foods (Kairenius *et al*., 2011).

B. proteoclasticus is the only known ruminal bacterium with the capacity to biohydrogenate 18-carbon PUFA to 18:0. Previous incubations with rumen fluid have established that 22:6*n*-3 inhibits the complete hydrogenation of 18-carbon PUFA causing *trans* 18:1 isomers to accumulate (AbuGhazaleh and Jenkins, 2004a). This is thought to be related to the toxic effects of 22:6*n*-3 on the growth and metabolic activity of *B. proteoclasticus* (Wasowska *et al*., 2006). However, *in vivo* studies have failed to show the relationship between 18:0 flow to the duodenum and *B. proteoclasticu*s DNA (Huws *et al*., 2010; Shingfield *et al*., 2012). Based on the findings of present study we suggest that *B. proteoclasticus* probably starts to hydrogenate 22:6*n*-3 before converting 18:1 isomers to 18:0. Biohydrogenation is

required to lower toxicity and therefore the reduction of products containing more double bonds are a greater priority which explains the lack of 18:1 reduction at time points where biohydrogenation of 22:6*n*-3 did occur. These findings possibly explain the lack of relationship of duodenal 18:0 to the duodenum and *B. proteoclasticu*s DNA (Huws *et al*., 2010; Shingfield *et al*., 2012).

CONCLUSIONS

B. proteoclasticus P18 is able to hydrogenate 22:6*n*-3 *in vitro*. The rate and extent of biohydrogenation depended on the initial concentration of 22:6*n*-3 and the duration of incubation. Several 22:5, 22:4, 22:3 and 22:2 isomers were identified. The products formed irrespective of the initial concentrations of 22:6*n*-3 and type of growth media suggests *B. proteoclasticus* P18 had a consistent pathway of 22:6*n*-3 hydrogenation. During the simultaneous presence of both 18:2*n*-6 and 22:6*n*-3, B*. proteoclasticus* P18 initiated 22:6*n*-3 metabolism before converting 18:1 isomers into 18:0. Under the current culture conditions, *B. fibrisolvens* D1 failed to hydrogenate 22:6*n*-3.

General Discussion

There is increasing evidence that polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (22:6*n*-3) provide physiological benefits to many species, including human and dairy cows (Baker *et al*., 2016; Cardoso *et al*., 2016; Swanson *et al*., 2012; Mattos *et al*., 2004). Therefore, a numbers of studies have examined the supplementation of 22:6*n*-3 sources in the diet. In ruminants, the 22:6*n*-3 available for absorption in the small intestine is limited due to biohydrogenation taking place in the rumen. Rumen biohydrogenation is the microbial saturation of dietary PUFA and is thought to be a detoxification mechanism of the bacteria against the inhibitory effects of PUFA (Henderson, 1973). At the start of the current PhD research, metabolic pathways of 22:6*n*-3 biohydrogenation and bacterial species involved were unknown, although it was well established that biohydrogenation of 22:6*n*-3 extensively occurs in the rumen in vivo (Lee *et al*., 2008; Shingfield *et al*., 2003) and during incubations with rumen fluid in vitro (AbuGhazaleh and Jenkins, 2004; Fievez *et al*., 2007). Hence, the main objective of this doctoral research project was to identify ruminal bacteria able to biohydrogenate 22:6*n*-3.

Two approaches have been used for this purpose. In one approach, we used mixed cultures of ruminal microorganisms as starting inoculum (Chapter 2) and in a second approach, available bacterial isolates were used (Chapter 3). It was through the latter that B. *proteoclasticus* P18 was found to be able to hydrogenate 22:6*n*-3 (Chapter 3). Unfortunately, none of the bacterial species identified from the mixed cultures were able to induce 22:6*n*-3 biohydrogenation. With both approaches, bacteria rather than protozoa were the target micro-organisms we focused on as the latter only play a

minor role in the known biohydrogenation pathways of 18:2*n*-6 and 18:3*n*-3 (linoleic and linolenic acid respectively), which was assumed to be similar for 22:6*n*-3.

22:6*n*-3 biohydrogenation pathway

Few studies have contributed to delineate the pathways of biohydrogenation of 22:6*n*-3. Previously, potential biohydrogenation intermediates were suggested after detailed analysis of ruminal and omasal digesta of ruminants fed fish oil or marine algae (both rich in 20:5n-3 and/or 22:6*n*-3) (Kairenius *et al*., 2011; Toral *et al*., 2012) from which the first scheme of the main 22:6*n*-3 biohydrogenation pathway was suggested (Figure 1) (Doreau *et al*., 2012). However, identification of their metabolic origin is extremely challenging because numerous 20- and 22-carbon FA showed a positive ruminal balance or accumulated to a greater extent in the rumen as compared with the non-supplemented controls. In these studies, intermediates with a conjugated double bound were not detected, but several intermediates with at least one *trans* double bound occurred, indicating hydrogenation of 22:6*n*-3 involved a series of *cis* to *trans* isomerizations, as well as reduction steps. By analogy with the known pathways of 18:2*n*-6 and 18:3*n*-3 metabolism in the rumen, it has been speculated that the initiation of ruminal hydrogenation of 22:6*n*-3 would yield a 22-carbon conjugated FA with 6 double bonds, containing at least one *trans* double bond (Jenkins *et al*., 2008). Nevertheless, in agreement with earlier studies (Doreau *et al*., 2012; Kairenius *et al*., 2011), in the current PhD, no indication was found of the formation of a 22-carbon conjugated FA (Chapter 3). This might be attributed to rapid biohydrogenation of the conjugated FA which could have resulted in the absence of any accumulation of the conjugated 22-carbon FA at the time points which have been

investigated here. Alternatively, this might also suggest that the 22:6*n*-3 biohydrogenation system does not involve an initial isomerization step. Metabolism of 22:6*n*-3 by pure culture with *B. proteoclasticus* resulted in the appearance of several 22-carbon FA eluting during the GC analysis between docosaenoic acid (22:0) and 22:6*n*-3. The intermediates produced comprises 12 different FA (Chapter 3). Three of those intermediates showed identical double bond positions as previously reported (Doreau *et al*., 2012) whereas the nine remaining ones are novel FA (Figure 1). In addition, metabolism of 22:6*n*-3 by *B. proteoclasticus* does not complete the saturation of 22:6*n*-3 to its final saturated product 22:0. In contrast, substantial amounts of 22:0 where formed by *in vitro* incubations with mixed cultures (Chapter 1). About 20% of the 22:6*n*-3 which disappeared had been recovered as 22:0, illustrating the possibility of rumen bacteria to form 22:0 from 22:6*n*-3. The current data cannot confirm that *B. proteoclasticus* is able to completely saturate 22:6*n*-3 to 22:0. However, as this bacteria is currently the only bacteria able to completely saturate 18:3*n*-3 and 18:2*n*-6 to 18:0, it might also be able to form 22:0 from 22:6*n*-3. A possible reason for this might be related to the time needed to saturate 22:6*n*-3 to 22:0. Extending the incubation time or reducing the concentration of 22:6*n*-3 might be options to investigate this, but depletion of nutrients and accumulation of volatile fatty acids resulting in a reduced pH might hamper this.

Several products formed by *B. proteoclasticus* were reported before in the literature. In order to further confirm that these products are produced by mixed cultures of microorganisms, samples obtained with mixed culture as in Chapter 1 could be analyzed. It is important that the design includes sampling at several time points to gain insight in the formation and metabolism of these intermediate products. In

addition, because a multitude of peaks are formed, these experiments are best performed with pure 22:6*n*-3 and not with a FA source containing several long-chain unsaturated fatty acids, such as fish oil. Indeed, fish oil contains next to 22:6*n*-3 also appreciable amounts of e.g. 20:5*n*-3, 21:5*n*-3, 22:5*n*-3, 22:5*n*-6 and *cis*-11 22:1 (Shingfield *et al*., 2012). As these fatty acids are probably also metabolized in the rumen, resulting in a multitude of products, it would be very difficult to indicate whether products are originating from 22:6*n*-3 or from other fatty acids added to the incubation flask.

Little is known on how the growth media affects the conversion of 22:6*n*-3, which is due to the lack of knowledge of bacterial species involved in this process. The results from this PhD suggest that *B. proteoclasticus* is able to biohydrogenate 22:6*n*-3, and that this the extent is dependent on the amount of 22:6*n*-3 present. In addition, hydrogenation occurred only after initiation of growth. Such observations were also made when demonstrating the capability of *B. proteoclasticus* P18 to form 18:0 from 18:2*n*-6 by Wallace et al. (2006). When 18:2*n*-6 was added to stationary-phase cells, CLA accumulated (predominantly as cis-9, trans-11 18:2) and no 18:1 or 18:0 was formed. However, adding 18:2*n*-6 before inoculation or in the exponential phase resulted in the formation of 18 1 and 18:0 (Wallace et al., 2006). From this, it can be hypothesized that changes in the media composition which affect the growth of *B. proteoclasticus* will affect conversion of 22:6*n*-3.

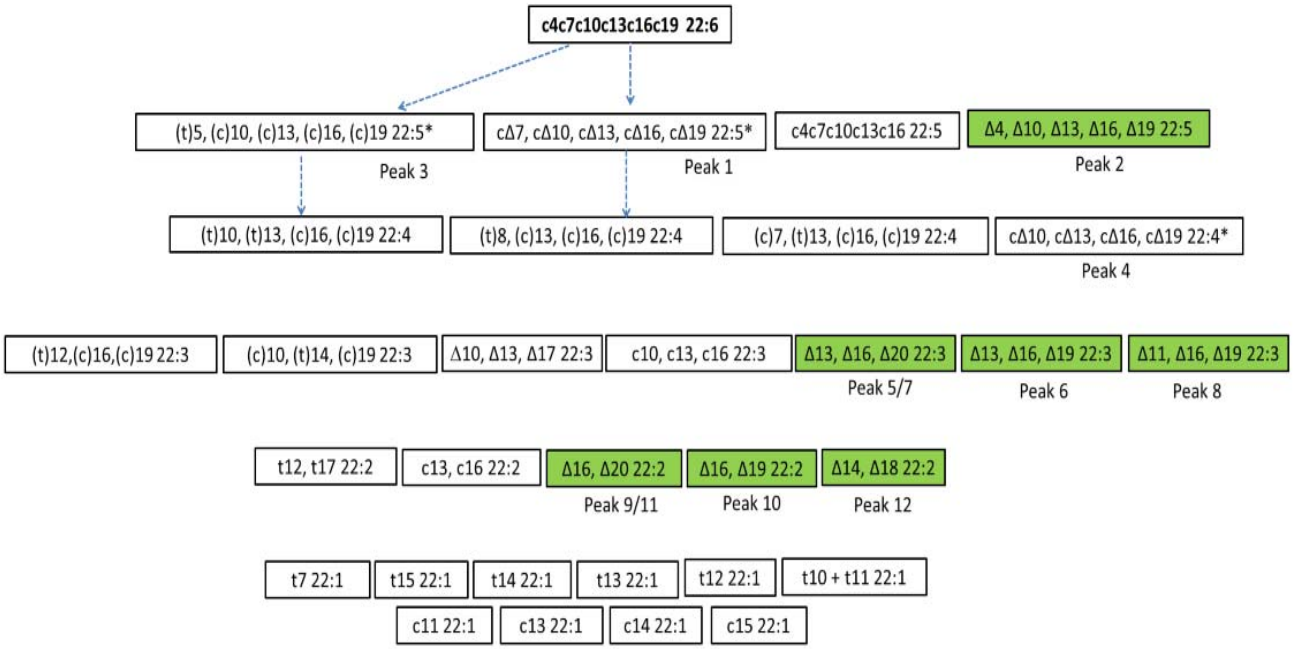

Figure 1. Putative intermediates formed in the biohydrogenation pathway of 22:6*n*-3 measured in ruminal and omasal digesta of ruminants fed fish oil or marine algae, adapted from Doreau et al. (2012) and including additional isomers formed by *B. proteoclasticus* as identified in Chapter 3. Letter preceding the double bond position of the FA indicate *trans* or *cis* geometry. (t or c respectively). Parenthesis on t or c indicate tentative geometry of double bonds. When geometry of double bond is unknown is represented by Δ (delta) symbol. * indicates intermediate identified in Chapter 3 with identical double bond positions to the intermediates presented in the putative pathway by Doreau et al. (2012). Highlighted in green indicates novel intermediates found in our study (Table 2 in Chapter 3) and numbers presented below boxes refer to identification in the GC chromatogram (Figure 2 in Chapter 3).

Identification of bacteria involved in 22:6*n*-3 biohydrogenation

As indicated before, two approaches have been used for this purpose, either starting from mixed rumen inoculum or using pure cultures. Below, some advantages and disadvantages of both approaches as well as specific difficulties of the methodologies for isolation of 22:6*n*-3 biohydrogenating bacteria are discussed.

Selective media

A common isolation technique when starting from mixed cultures is the use of selective media. Selective media provide one or a limited number of substrates that the target microbes use as source of energy resulting in the growth of only a small number of species (Kenters *et al*., 2011; Wang *et al*., 2010). Several examples of this isolation approach can be found in the literature. For instance addition of cellulose as sole carbon source allowed to isolate cellulolytic bacteria from the rumen (Wang *et al*., 2010). In a similar approach methanogens, which utilize hydrogen and CO_2 or formate, were isolated from the rumen (Rea *et al*., 2007; Smith and Hungate, 1958). Another example is fructooligosaccharide-utilizing bacteria isolation from faeces using these sugars as selective substrate (Sghir *et al*., 1998). What is specific here, is that the bacteria of interest, which grow on these selective media, are able to generate ATP from compounds present in these media whereas the bacteria of less interest are not able to generate ATP. This requirement hampers the usefulness of this technique to isolate hydrogenating bacteria because they do not generate ATP from the biohydrogenation of PUFA. Conversely, the mechanism of adding hydrogen to a double bond is energetically costly because it is NADH dependent (Hunter *et al*.,

1976). Moreover, known biohydrogenating bacteria are shown to have the greatest sensitivity towards PUFA (Maia *et al*., 2007), which causes an additional difficulty when attempting to develop strategies to isolate hydrogenating bacteria. Research on designing the medium composition (in terms of nutrients) ultimately may lead to obtain a medium which is selective or at least stimulating for 22:6*n*-3 biohydrogenating bacteria. However, the nutrients of preference of known biohydrogenating bacteria are not specific. For instance, *B. fibrisolvens* - the most well-known biohydrogenating bacterium - typically ferments a wide range of substrates including maltose, sucrose, cellobiose and cellulose but does not utilize lactate or glycerol; whereas *Propionibacterium acnes,* which is also a hydrogenating bacteria, uses preferentially lactate over glucose (Moore and Cato, 1963; Wallace et al., 2006). On the other hand, the biohydrogenating bacterium *B. proteoclasticus* P-18 utilizes a broad range of sugars including glucose, fructose, galactose but does not utilize cellulose or lactate (Wallace *et al*., 2006). This illustrates, that substrate preferences can vary between different species and strains of known biohydrogenating bacteria. Hence, the identification of a selective medium would require to test a huge number of substrate sources. In addition, medium components such as minerals, vitamins, peptides, amino acids and other micro-elements might play a role in the metabolic activity of biohydrogenating bacteria. Basically, every component of the medium other than water could affect the metabolic activity of bacteria. Accounting for these effects by investigating every component of the medium can be a daunting task. Accordingly, this approach has not been a methodology of choice in the current PhD research.

Screening pure cultures

An alternative to identify bacteria capable of hydrogenating 22:6*n*-3, is to screen pure cultures of rumen bacteria. These bacteria then should be exposed to 22:6*n*-3 and its disappearance is then monitored. However, in earlier pure culture studies this approach failed as the most well-known biohydrogenating bacteria were not growing in the presence of 22:6*n*-3 putatively due to its high toxicity (Maia *et al*., 2007, 2010). Hence, a first requirement to allow application of the pure culture technique is the reduction of the PUFA toxicity. Because of the colloidal properties of FA, their toxicity has been partially linked to their capacity to adhere to bacteria. Hence, providing alternative adsorption places could reduce the amount of PUFA per unit of bacterial biomass. In this PhD, two different systems employing particulate material were tested for their impact on biohydrogenation of 22:6*n*-3: 1/ addition of adsorbant compounds (Chapter 1) and 2/ addition of rumen fluid particles (Chapter 2). Such toxicity-reducing measures were successful to enhance the growth of the most well-known active biohydrogenating bacteria (*B. fibrisolvens* and *B. proteoclasticus*) in the presence of quantifiable amounts of 22:6*n*-3. Using these adaptations to the culture media, we were the first to show that *B. proteoclasticus* is able to metabolize 22:6*n*-3 in a dose dependent manner. The latter also indicates the importance of lowering the amounts of 22:6*n*-3 in alleviating 22:6*n*-3 toxicity (Chapter 1). Obviously, one might proceed to screen pure cultures to identify other bacteria responsible of 22:6*n*-3 biohydrogenation. However, to test a large number of species besides the well-known rumen biohydrogenating strains, cumbersome and time consuming experiments would be needed.

Dilution to extinction

Hence, an alternative approach which does not require selective media nor screening of pure cultures, is the dilution-to-extinction approach as described by Kenters et al. (2011), in which isolation from mixed cultures was carried out by serially diluting the inoculum to obtain one single cell (Kenters *et al*., 2011). In their method, about 1000 tubes were inoculated with diluted rumen fluid in order to introduce a mean of ≤ one culturable cell. After growth and sub-culturing, they achieved 54 pure cultures. A drawback of this technique is that the abundant bacteria in rumen fluid are more likely to be present in the diluted tubes. Hence, a large number of tubes is probably needed to isolate less abundant species. Obviously using a less complex inoculum as starting inoculum might result in isolating less abundant bacteria. Accordingly, an alternative interpretation of this method is the enrichment through dilution. This method consists of serial dilutions of the inoculum using a standard medium with supplementation of the substrate of interest. Other authors used this dilution-to-enrichment method to isolate a microbial consortium from rumen liquor that degrades cellulose (Wang *et al*., 2010). The most diluted inoculum that effectively degraded cellulose constituted the enrichment. The method facilitated the identification of the bacteria because the diluted inoculum consisted mainly of three stains. Hence, in a similar way, the dilution-to-enrichment approach was proposed to simplify the complexity of mixed cultures of microorganisms to a simple consortia responsible for biohydrogenation of 22:6*n*-3 by serial dilution. Just as for the pure culture experiments, also this approach would not have been possible if we were not able to reduce the toxicity problem since already the small reduction of inoculum to 5 % v/v showed little biohydrogenation in the absence of uncentrifuged-autoclaved rumen fluid (Chapter 2). In the current PhD we combined the adjusted media with

uncentrifuged-autoclaved rumen fluid, with the dilution-to-enrichment approach and each dilution was tested for biohydrogenation of 22:6*n*-3. Disappearance of 22:6*n*-3 decreased with increasing inoculum dilution and this was accompanied with changes in the microbial community with some bacteria becoming more abundant whereas other bacteria disappeared. Differences between the bacterial community in the dilution where 22:6*n*-3 disappearance still occurred and the dilution in which 22:6*n*-3 did not disappear anymore were of particular interest. After identification, pure cultures of these bacteria were tested for their 22:6*n*-3 biohydrogenation capacity. Unfortunately, for none of the strains tested their capacity to biohydrogenate 22:6*n*-3 was confirmed (Chapter 2).

Alternative high throughput method: single cell encapsulation

Another potentially efficient approach for the isolation of bacteria can be achieved by high throughput methods such as the single cell encapsulation (SCE) (Wang and Bodovitz, 2010). SCE has been introduced in the fields of life science, diagnostics, the pharmaceutical industry and renewable energy (El-Ali *et al*., 2006; Yin and Marshall, 2012). In microbiology, SCE has been used to isolate single microbes from complex populations living in sea waters and soil (Ishii *et al*., 2010). The method uses devices to manipulate small amounts of liquid by passing the liquid through small channels that are comparable to the size of a single cell (∼10 μm in size and roughly ∼1 pL in volume) and catch individual bacterial cells in a gel capsule (Whitesides, 2006). The method enables high throughput generation of single cells in microdroplets (Yin and Marshall, 2012). For our purposes of identification of biohydrogenating bacteria this procedure might help to isolate bacteria which could

then be tested for biohydrogenation. Manipulation of these droplets to release the cell from the enclosed capsule and their subsequent growth to be assessed for biohydrogenation requires additional investigations. Of even more interest would be if the disappearance of 22:6*n*-3 could be assessed immediately in the single cell microdroplets. However, this additionally would require a high-throughput method which would allow to monitor the metabolism of extremely low amounts of 22:6*n*-3. Some analytical challenges related to the assessment of the 22:6*n*-3 disappearance are discussed in the next paragraph.

Assessing 22:6*n*-3 metabolism

In order to illustrate biohydrogenation, FA have to be quantified. During the current PhD, this quantification was carried out according to the procedure of Vlaeminck et al. (2014). The procedure involved several steps including freeze drying of the incubation samples, acid-base catalyzed methylation, and further determination by gas chromatography. The whole process of FA analysis involved important amounts of reagents which makes it costly and time consuming. Accordingly, this analysis represented an additional constraint to isolation or culture techniques which typically generate a large number of samples. To allow a routine analysis of a large number of samples a more optimized high-throughput system for FA analysis is needed. Fast high-throughput methods can be based on spectrophotometry. Regarding FA analysis, an example is the measurement of CLA production by employing UV spectrophotometry (Barrett *et al.*, 2007). In this example, researchers were interested in screening several numbers of bacterial cultures for their production of conjugated

linoleic acid (CLA). CLA is a FA that contains a conjugated double bond in its structure. The detection of CLA was resolved when found that the conjugated double bonds can be detected with high accuracy at 233 nm wavelength eliminating the need for gas chromatography (Rodriguez-Alcala *et al*., 2011; Wang *et al*., 2007). In this way screening a large number of cultures for CLA production has been simplified. It was speculated before that during the process of 22:6*n*-3 biohydrogenation, the initial product contains a conjugated bond (Jenkins *et al*., 2008). If so, this might allow measuring the disappearance of 22:6*n*-3 by the appearance of conjugated FA. However, evidence presented by Kairenius et al., (2011) and in this thesis (Chapters 1 and 3) indicate that a conjugated FA is not the initial product of hydrogenation of 22:6*n*-3 or at least, no conjugated FA is detected. It is not clear whether 22:6*n*-3 or ultimately its intermediates can be measured spectrophotometrically but if such a system is available it would considerably facilitate the experiments towards identification of biohydrogenating bacteria.

Application of uRF in research towards fatty waste waters from industrial processes

Long chain fatty acids (LCFA) are produced during the hydrolysis of oils and fats and are commonly present in fatty waste waters particularly form the food industry (Appels *et al.,* 2011). Interest towards bacterial metabolism of LCFA has been significant in industrial facilities making use of such waste waters. Indeed, these waste waters have a high methane production potential, which is of particular interest in biogas production plants. However, a high lipid load might impair the exploitation of

the biogas plant because of the toxic effects of LCFA towards the bacteria used as inoculum to digest those wastes (Rinzema *et al.,* 1994). Studies dealing with LCFA toxicity in biogas plants used several strategies to recover the digestion system from LCFA inhibitory effects, such as the solubilization of lipid waste via saponification or enzymatic pre-treatments, the application of feeding procedures based on sequential LCFA accumulation steps, the addition of easily degradable co-substrates or the addition of inorganic agents (Palatsi *et al*., 2010). From the results reported in Chapter 2, one might suggest the addition of uncentrifuged-autoclaved rumen fluid (uRF) as an alternative strategy to enhance digestion in such waste water systems. However, towards practical applications, this might be a relatively cumbersome procedure since rumen fluid for such industrial scale processes would need to be collected from slaughter houses. Nevertheless, uRF could be useful for laboratory or pilot tests towards strategies to enhance detoxification of LCFA and increase biogas production. If addition of uRF would successfully increase biogas production, the reason for this stimulation could be further investigated and 'artificial replacers' for uRF could be implemented at industrial level. Although the precise cause of the stimulatory effect of uRF was not elucidated during this PhD, we hypothesized that the fibers present in uRF could be one reason for this effect providing additional fermentation sources or reducing the inhibitory effects of PUFA towards bacteria by adsorption (Chapter 1). In previous research on biogas reactors using cow manure as inoculum, it was anticipated that fibers present in the manure could have an adsorptive effect to overcome LCFA toxicity (Palatsi *et al*., 2010). Addition of fibers obtained from filtered digested manure had a positive effect on fermentation activity in the presence of a LCFA mixture.

Additionally, the methodological approach applied during this PhD, could be used in studies towards 'key microbes' required for successful biogas production from waste waters with a high LCFA load. Indeed, the biogas industry makes use of several sources of microbial inocula in order to find the most effective system to produce biogas; for instance, inocula originating from granular sludge or manure, but also the introduction of artificially assembled microbial communities also has been considered to optimize and better standardize the biogas production system (Cavaleiro *et al*., 2010; De Vrieze *et al*., 2015). Similar to the culture techniques used in this thesis to stimulate bacteria able to metabolize 22:6*n*-3 (Chapter 2), the strategy to use uRF in an experimental set-up also might allow to enrich the microbial community able to detoxify the LCFA in food waste waters. This approach could also be used when assembling industrial inoculum for biogas production.

Furthermore, *B. proteoclasticus*, the bacterial species identified during the current PhD research is (up till now) the sole candidate to biohydrogenate 22:6*n*-3 (Chapter 3). As 22:6*n*-3 can be considered as one of the most toxic PUFA (Desbois and Smith 2010), this species might be of particular interest to enrich e.g. in (artificial) inocula of biogas plants relying on fatty waste waters. Indeed, the ability of *B. proteoclasticus* to convert this toxic PUFA might open perspectives for inocula enriched in this species to stimulate methane production in biogas plants using waste waters through enhanced detoxification of LCFA. Enrichment success obviously will depend on the survival of the inoculated microbial culture (Jiang *et al*., 2007). Therefore, the applicability and limits of enrichment with this bacterial species can be initially tested in batch assays.

Conclusion

Ruminal biohydrogenation is an important metabolic process to the rumen microbial ecosystem with direct consequences on the fatty acid profile of ruminant products such as milk, dairy products and meat, and ultimately to human health. Feeding strategies to improve the healthiness of ruminants-derived products are developed. Supplementation of 22:6*n*-3 food sources to ruminant diets was one of the strategies used in previous years. However, a better understanding of the bacterial species involved in 22:6*n*-3 biohydrogenation in the rumen is necessary to allow to manipulate ruminal biohydrogenation to improve the fatty acid profile of ruminant meat and milk in a manner favorable to human health. Overall, the present PhD research showed addition of particles (as uncentrifuged rumen fluid) allowed metabolism of 22:6*n*-3 in highly diluted rumen fluid. Using this approach, putative candidates for 22:6*n*-3 biohydrogenating bacteria were identified. Future research should confirm whether these candidates are actually involved in biohydrogenation of 22:6*n*-3. Moreover, a pure culture study revealed the first bacterial species able to biohydrogenate 22:6*n*-3 (*B. proteoclasticus*) and provided an insight on the products formed during the metabolism on 22:6*n*-3 by this species. This bacteria could serve as a model organism to provide details on the formation of biohydrogenation intermediates and the construction of a pathway of 22:6*n*-3 biohydrogenation in future research.

Summary

Ruminant derived products such as milk and meat are important sources of dietary fat in the human diet, however the fat composition of these products is high in saturated fatty acids (SFA) and poor in polyunsaturated fatty acids (PUFA). Altering the fatty acid composition of milk and meat represent one means to lower the human intake of SFA and increase PUFA. One of the PUFA that appears desirable is 22:6*n*-3 because of its association with health promoting effects in humans as well as in the ruminant. In this regards, several studies have examined the supplementation of 22:6*n*-3 sources as feeding strategy to increase 22:6*n*-3 content in ruminant products. Yet, the increase in 22:6*n*-3 concentrations in milk fat and intramuscular fat is small, particularly due to biohydrogenation occurring in the rumen. Biohydrogenation in the rumen is a well-known phenomenon by which unsaturated fatty acids are gradually saturated by bacteria residing in the rumen. Although biohydrogenation of 22:6*n*-3 occurs extensively in the rumen, both the bacterial species involved as well as the metabolic pathways of this biohydrogenation process remain unknown.

Therefore, the main objective of this doctoral research project was to identify ruminal bacteria involved in the biohydrogenation of 22:6*n*-3 and to characterize fatty acids formed during this process. For this, two different approaches were followed. In one approach, pure cultures of rumen bacteria were used to test their capacity for 22:6*n*-3 biohydrogenation. In a second approach, rumen fluid containing mixed cultures of rumen bacteria were investigated to identify the bacteria involved in 22:6*n*-3

biohydrogenation using a dilution-to-enrichment approach in combination with molecular techniques.

However, a first challenge to overcome, prior to the application of these two approaches, was the particular sensitivity of biohydrogenating bacteria to the toxic effects of 22:6*n*-3, among others by the adherence to bacterial cells. Thus, in order to reach the objectives of this PhD research, reduction of the toxicity of 22:6*n*-3 was initially investigated. For this, adjustments to the incubation conditions had to be made to lower the adverse effects of 22:6*n*-3 *in vitro*. Addition of substances such as mucin and arabic gum to the culture medium provided an alternative site for adsorption of 22:6*n*-3 and as such decrease the amount of 22:6*n*-3 available to adhere to bacterial cells. Accordingly, these substances reduced the inhibitory effects on bacterial growth and metabolic activity and increased the disappearance of 22:6*n*-3 during a 24-h *in vitro* assay (Chapter 1). However, as both mucin and gum arabic also provided fermentable substrate for ruminal bacteria it could not be excluded that the enhanced supply of fermentable substrate rather than their adsorbing capacity was the main reason for the increased disappearance of 22:6*n*-3. Hence, additional experiments were performed in which mucin and gum arabic were replaced by equal amounts of starch, cellulose and xylan, which are assumed to have no or limited adhesion properties. Across all additives, disappearance of 22:6*n*-3 particularly related to volatile fatty acid production, suggesting that the stimulatory effect of mucin and gum arabic on disappearance of 22:6*n*-3 might have been particularly related to stimulation of bacterial growth rather than provision of an alternative site for adsorption.

Mucin and gum arabic not only stimulated the disappearance of 22:6*n*-3 but at low initial concentrations of 22:6*n*-3 in the incubation media (0.02 mg/mL) also enhanced the formation of 22:0. The confirmation of complete conversion of 22:6*n*-3 to 22:0 by ruminal batch cultures in this research was of particular interest as former studies suggested no or only trace formation of this end product. In our research, up to 20% of the 22:6*n*-3 was reduced to 22:0, indicating that the mixed rumen microorganisms are capable to extensively reduce all six double bonds. The successful *in vitro* metabolism of 22:6*n*-3 was a first and essential prerequisite for the further studies within the frame of this PhD.

In a second part of this work, a dilution-to-enrichment experiment in combination with molecular techniques were used to identify potential bacterial candidates involved in the biohydrogenation of 22:6*n*-3. The addition of autoclaved-uncentrifuged rumen fluid (uRF) to incubation fluid containing highly diluted inoculum seemed necessary to stimulate the biohydrogenation of 22:6*n*-3 (Chapter 2).

Disappearance of 22:6*n*-3 decreased with increasing inoculum dilution and this was accompanied with changes in the microbial community with some bacteria becoming more abundant whereas other bacteria disappeared. Differences between the bacterial community in the dilution where 22:6*n*-3 disappearance still occurred and the dilution in which 22:6*n*-3 did not disappear anymore were investigated. In this way, species of interest were found to belong to *Streptococcus*, *Selenomonas* and unclassified bacteria. Identified species were then tested for their 22:6*n*-3 biohydrogenation capacity for the commercially available isolates of Streptococcus and Selenomonas. Two Selenomonas strains (S. *ruminantium DSM 2150* and S. *lactilytica* DSM 2872) and one *Streptococcus* strain (S. *gallolyticus* DSM 16831) were

used for this purpose but did not show biohydrogenation of 22:6*n*-3 at the conditions used. However, their growth was not impaired at any of the 22:6*n*-3 concentrations (Chapter 2).

Finally, metabolism of 22:6*n*-3 by pure cultures of rumen bacteria was investigated (Chapter 3). Two rumen bacteria were used, *Butyrivibrio fibrisolvens* D1 and *Butyrivibrio proteoclasticus* P18 which are the most well-known bacteria hydrogenating 18:2*n*-6 and 18:2*n*-3. B. *fibrisolvens* failed to hydrogenate 22:6*n*-3 *in vitro*. However, B. *proteoclasticu*s was able to metabolize 22:6*n*-3. The rate and extent of biohydrogenation depended on the initial concentration of 22:6*n*-3 and the duration of the incubation. Gradual appearance of intermediates indicated that biohydrogenation of 22:6*n*-3 by B. *proteoclasticu*s occurred by pathways of hydrogenation and isomerization resulting in a variety of 22-carbon unsaturated FA.

In conclusion, B. *proteoclasticu*s is so far, the only bacteria known to biohydrogenate 22:6*n*-3 and it could serve as a model organism to provide details on the formation of biohydrogenation intermediates and the construction of a pathway of 22:6*n*-3 biohydrogenation in future research. Adjustment of the incubation conditions with addition of substances are key features to stimulate biohydrogenating of 22:6*n*-3 by mixed and pure cultures when aiming at studying the biohydrogenation pathways and bacteria involved in this process. Future studies on isolation/identification of hydrogenating bacteria should focus on high throughput methods. A better understanding of the bacterial species involved in 22:6*n*-3 biohydrogenation in the rumen may allow to manipulate ruminal biohydrogenation to improve the fatty acid profile of ruminant meat and milk in a manner favorable to human health.

Samenvatting

Herkauwerproducten zoals melk en vlees zijn belangrijke vetbronnen van het menselijke dieet, maar deze producten zijn rijk aan verzadigde vetzuren en arm aan meervoudig onverzadigde vetzuren. De vetzuursamenstelling van melk en vlees aanpassen is één middel om de opname van verzadigde vetzuren te doen dalen en die van meervoudig onverzadigde vetzuren te doen toenemen. Eén van de belangrijkste meervoudig onverzadigde vetzuren vanwege de associatie met gezondheidsbevorderende effecten bij zowel mens als herkauwer in dit verband is docosahexaeenzuur (22:6*n-3*). Verschillende studies onderzocht reeds eerder de toediening van producten rijk aan 22:6*n-3* als voedingsstrategie om het 22:6*n-3* gehalte in herkauwerproducten te doen verhogen. Toch is de toename in 22:6*n-3* concentraties in melkvet en intramusculair vet bij herkauwers eerder klein, vooral door biohydrogenatie in de pens. Biohydrogenatie in de pens is een bekend verschijnsel waarbij onverzadigde vetzuren geleidelijk verzadigd worden door pensbacteriën. Hoewel biohydrogenatie van 22:6*n-3* uitgebreid optreedt in de pens, blijven zowel de verantwoordelijke bacteriën alsmede de metabole processen van deze biohydrogenatie onbekend.

De belangrijkste doelstelling van dit onderzoeksproject is daarom om de pensbacteriën betrokken bij de biohydrogenatie van 22:6*n-3* te identificeren en vetzuren gevormd tijdens dit proces te karakteriseren. Hiervoor werden twee verschillende benaderingen gevolgd. In een eerste benadering werden zuivere culturen van pensbacteriën gebruikt om hun biohydrogenatiecapaciteit van 22:6*n-3* te testen. In een tweede benadering werd pensvloeistof met gemengde culturen van

pensbacteriën onderzocht om de bacteriën betrokken bij de 22:6*n-3* biohydrogenatie te identificeren aan de hand van een verdunning-tot-aanrijking benadering in combinatie met moleculaire technieken.

Vóór het testen van deze twee benaderingen diende een eerste uitdaging te worden overwonnen, namelijk de gevoeligheid van deze bacteriën voor de toxische effecten van 22:6*n-3*, die onder meer veroorzaakt wordt door de aanhechting van 22:6*n-3* aan bacteriële cellen. Teneinde de doelstellingen van dit doctoraatsonderzoek te bereiken werd de vermindering van de toxische effecten van 22:6*n-3* tijdens incubatie eerst onderzocht. Hiervoor moesten aanpassing worden gemaakt aan de reguliere incubatie-omstandigheden om de nadelige effecten van 22:6*n-3 in vitro* te verlagen. Toediening van stoffen zoals mucine en Arabische gom aan het kweekmedium verschafte een alternatieve locatie voor adsorptie van 22:6*n-3* en verlaagde als dusdanig de hoeveelheid aan 22:6*n-3* beschikbaar voor adsorptie aan bacteriële cellen. Deze stoffen verlaagden de remmende werking van 22:6*n-3* op bacteriegroei en metabolische activiteit en verhoogde als dusdanig de biohydrogenatie van 22:6*n-3* gedurende een 24 h *in vitro* assay (Hoofdstuk 1). Zowel mucine als Arabische gom voorzien echter ook fermenteerbaar materiaal voor de pensbacteriën, waardoor het niet kon worden uitgesloten dat de verhoogde aanvoer van fermenteerbare materie en niet de adsorberende capaciteit de belangrijkste reden voor de verhoogde verdwijning van 22:6*n-3* was. Een nieuw experiment werd vervolgens uitgevoerd waarbij mucine en Arabische gom vervangen werd door gelijke hoeveelheden aan zetmeel, cellulose en xylaan, waarvan werd verondersteld dat ze geen of beperkte hechtingseigenschappen hebben. De verdwijning van 22:6*n-3* over alle additieven heen was gerelateerd aan de vluchtige vetzuurproductie, wat

suggereert dat het stimulerende effect van mucine en Arabische gom op de verdwijning van 22:6*n-3* eerder zou kunnen verband houden met de bevordering van bacteriegroei dan het aanreiken van een alternatieve locatie voor adsorptie.

Mucine en Arabische gom bevorderden niet alleen het verdwijnen van 22:6*n-3*, maar ook de verhoogde vorming van het verzadigde vetzuur 22:0 bij lage startconcentraties aan 22:6*n-3* in het incubatiemedium (0.02 mg/mL). De bevestiging van een volledige omzetting van 22:6*n-3* tot 22:0 door pensculturen in dit onderzoek was van bijzonder belang aangezien eerdere studies geen of slechts een summiere vorming van dit eindproduct suggereerden. Ons onderzoek gaf aan dat tot 20% van 22:6*n-3* werd gereduceerd tot 22:0, wat aangeeft dat een mengcultuur aan micro-organismen uit de pens wel degelijk alle zes de dubbele bindingen kunnen reduceren. Deze succesvolle *in vitro* omzetting van 22:6*n-3* was een eerste en essentiële voorwaarde voor de verdere experimenten in het kader van dit doctoraat.

In het tweede deel van dit werk werd een verdunning-tot-aanrijking experiment in combinatie met moleculaire technieken gebruikt om potentiële bacteriën betrokken bij de biohydrogenatie van 22:6*n-3* te identificeren. De toevoeging van geautoclaveerd en ongecentrifugeerd pensvloeistof (uRF) aan incubatie medium met sterk verdund inoculum bleek noodzakelijk om de biohydrogenatie van 22:6*n-3* te stimuleren (Hoofdstuk 2).

De verdwijning van 22:6*n-3* nam af bij toenemende verdunning van het inoculum en ging gepaard met veranderingen in de microbiële gemeenschap, waarbij bepaalde bacteriën overvloediger aanwezig werden, terwijl andere bacteriën verdwenen. Verschillen tussen de bacteriële gemeenschappen bij de verdunning waarbij verdwijning van 22:6*n-3* nog optrad enerzijds en de verdunning waarbij geen 22:6*n-3*

meer verdween anderzijds werd onderzocht. Op deze manier werd ontdekt dat *Streptococcus*, *Selenomonas* en ongeclassificeerde bacteriën tot de soorten van belang bleken te behoren. Commercieel beschikbare isolaten van de geïdentificeerde soorten *Streptococcus* en *Selenomonas* werden vervolgens getest op hun 22:6*n-3* biohydrogenatie capaciteit. Twee *Selenomonas* stammen (*S. ruminantium* DSM 2150 en *S. lactilytica* DSM 2872) en één *Streptococcus* stam (*S. gallolyticus* DSM 16831) werden gebruikt, maar biohydrogenatie van 22:6*n-3* werd niet waargenomen bij de gebruikte omstandigheden. Hun groei werd echter niet beïnvloed door de geteste 22:6*n-3* concentraties (Hoofdstuk 2).

Tot slot werd het metabolisme van 22:6*n-3* door pure culturen van pensbacteriën onderzocht (Hoofdstuk 3). Twee van de bekendste bacteriën verantwoordelijk voor de hydogenering van linolzuur (18:2*n-6*) en linoleenzuur (18:3*n-3*) werden hiervoor gebruikt, namelijk *Butyrivibrio fibrisolvens* D1 en *Butyrivibrio proteoclasticus* P18. *B. fibrisolvens* was niet in staat om 22:6*n-3 in vitro* te hydrogeneren, terwijl *B. proteoclasticus* hier wel toe in staat was. De snelheid en mate van biohydrogenatie was afhankelijk van de beginconcentratie aan 22:6*n-3* en de duur van de incubatie. Gedurende de incubatie verscheen geleidelijk een waaier aan verschillende 22-koolstof onverzadigde vetzuur tussenproducten, die aangaven dat biohydrogenatie van 22:6*n-3* door *B. proteoclasticus* optrad door hydrogenering en isomerisatie.

Samengevat, *B. proteoclasticus* is tot nu toe de enige geïdentificeerde bacterie die in staat is tot biohydrogenatie van 22:6*n-3* en zou als modelorganisme kunnen dienen om verdere details te kunnen verschaffen omtrent de vorming van biohydrogenatietussenproducten. Op basis hiervan zou in de toekomst het biohydrogenatietraject van 22:6*n-3* afgebakend kunnen worden. Aanpassing aan de

incubatie-omstandigheden door toevoeging van verscheidene stoffen is een belangrijk kenmerk om biohydrogenatie van 22:6*n-3* door gemengde en zuivere kweken te stimuleren bij experimenten gericht op het bestuderen van de biohydrogenatie paden en bacteriën die betrokken zijn bij dit proces. Verdere studies omtrent de isolatie en identificatie van bacteriën met hydrogenerende capaciteiten moeten zich richten op high-throughput methodes. Een beter begrip van de bacteriële species die verantwoordelijk zijn voor de biohydrogenatie van 22:6*n-3* in de pens kan helpen om de biohydrogenatie in de pens zodanig te manipuleren om het vetzuurprofiel van vlees en melk afkomstig van herkauwers in gunstige zin te verbeteren voor de menselijke gezondheid.

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Publications

International peer viewed articles (A1)

1. **Escobar, M., Vlaeminck, B., Jeyanathan, J., Thanh, L.P., Shingfield, K.K., Wallace R.J. and Fievez, V.** 2016. Effect of adsorbants on in vitro biohydrogenation of 22:6n-3 by mixed cultures of rumen microorganisms. Animal, 10:9, 1439–1447.

2. **Jeyanathan, J., Escobar, M., Wallace, R.J., Fievez, V. & Vlaeminck, B.** 2016. Biohydrogenation of 22:6n-3 by Butyrivibrio proteoclasticus P18. BMC Microbiology, 16, 104.

Conference articles (A3)

1. **Escobar, M., B. Vlaeminck, J. Jeyanathan, and V. Fievez,** 2016. Addition of uncentrifuged-autoclaved rumen fluid allows microbial biohydrogenation of 22:6n-3 in highly diluted rumen inoculum. 10th Joint symposium INRA-ROWETT. Clermont-Ferrand, France. Page 72.

2. **J. Jeyanathan, Escobar, M., R. J Wallace, V. Fievez and B. Vlaeminck.** 2016. Biohydrogenation of docosahexaenoic acid by Butyrivibrio proteoclasticus P18. 10th Joint symposium INRA-ROWETT. Clermont-Ferrand, France. Page 77.

Conference Proceedings (C1)

1. **Escobar, M., B. Vlaeminck, J. Jeyanathan, and V. Fievez,** 2016. Addition of uncentrifuged-autoclaved rumen fluid allows microbial biohydrogenation of 22:6n-3 in highly diluted rumen inoculum. Wageningen University. Proceedings of the 41th Animal Nutrition Research Forum; page 29-30.

2. **Escobar, M., B. Vlaeminck, K. J. Shingfield, R. J Wallace, and V. Fievez.** 2015. Effect of adsorbents on ruminal mixed culture interaction with DHA and its biohydrogenation in vitro. 20th National Symposium for Applied Biological Sciences. Universite Catholique de Louvain, 30th January. Comm. Appl. Biol. Sci, 80 (1), 151-155.

3. **Escobar, M., B. Vlaeminck, K. J. Shingfield, R. J Wallace and V. Fievez**. 2014. Effect of adsorbents on in vitro biohydrogenation of 22:6n-3 by mixed cultures of rumen microorganisms. Utrecht University. Proceedings of the 39th Animal Nutrition Research Forum; page 11-12.

Oral presentations

1. **Escobar, M., B. Vlaeminck, J. Jeyanathan, and V. Fievez**. Addition of uncentrifuged-autoclaved rumen fluid allows microbial biohydrogenation of 22:6n-3 in highly diluted rumen inoculum. Lipids in ruminants. (Lokeren, Belgium, 23rd September 2016).

2. **Escobar, M., B. Vlaeminck, J. Jeyanathan, and V. Fievez.** Addition of uncentrifuged-autoclaved rumen fluid allows microbial biohydrogenation of 22:6n-3 in highly diluted rumen inoculum. Animal Nutrition Research Forum. (Wageningen, The Netherlands, 15th April 2016).

3. **Escobar, M., B. Vlaeminck, K. J. Shingfield, R. J Wallace, and V. Fievez.** Effect of adsorbents on ruminal mixed culture interaction with DHA and its biohydrogenation in vitro. 20th National Symposium for Applied Biological Sciences. (Leuven, Belgium, 30th January 2015).

4. **Escobar, M., B. Vlaeminck, K. J. Shingfield, R. J Wallace and V. Fievez**. Effect of adsorbents on in vitro biohydrogenation of 22:6n-3 by mixed cultures of rumen microorganisms. Animal Nutrition Research Forum (Utrecht, the Netherlands, 3rd April 2014).

Teaching activities

- Responsible for practical exercises for the course “Animal Production Biology” (Prof. Veerle Fievez) (2015-2016)

- Tutor of Master student (Human Nutrition) with the thesis “Investigation of biohydrogenating bacteria of 22:6*n*-3 in goats with prenatal supplementation of 22:6n-3”.

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